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Developments in the Production of Mushroom Mycelium in Submerged Liquid Culture

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Summary. The process for the production of mushroom mycelium for food purposes has great potentiality. It would offer a simple, mechanized, inexpensive method for producing a nutritious food. Mushroom mycelium can be cultivated in submerged liquid culture on simple carbohydrate and nitrogen compounds with mineral salts. Yields are high and no special production problems are involved. Those species reported to have flavour are *Agaricus campestris*, *Morchella crassipes*, *Lepiota rachodes*, and *Coprinus comatus*. The mycelial growth is usually either in ball form or dispersed. The dispersed form, characterized by fine mycelium and secondary spores, may be a physiological mutation which gives greater yield but less flavour. Mycelium grown on solid media is said to have greater flavour than mycelium grown in submerged culture. Several methods for enhancing the flavour of the mycelium have been investigated. The taste of the mycelium has been variously reported from flavourless to equivalent or even preferable to that of the fruiting bodies. The fact remains, however, that it is only the problem of taste that has kept the process from commercial exploitation.

Introduction

There has been considerable interest in the production of mushroom mycelium ever since the first report of a process about a decade ago.[1] If successful, such a process would offer a new and revolutionary method of food production. The mycelium, like the fruiting body of the mushroom, has been shown to be valuable nutritionally as a source of amino acids and B-complex vitamins.[2–4] The process is rapid, taking as little as 30 hours from start to completion,[4] as compared with several months for the conventional method of mushroom production. Many of the large antibiotic producers, fermentation companies, and brewers have been interested, since their large tanks and other equipment for the propagation and handling of micro-organisms in quantities

could be utilized for producing mushroom mycelium. The product is uniform, requires no hand labour, and its production can be completely mechanized. It is obvious from these considerations that the mycelium would be only a fraction of the cost of a similar quantity of mushrooms produced by the traditional method. Furthermore, in the production of the mycelium, one would not be limited by the size or the appearance of the mushrooms, but might produce many different species to yield different flavours. The mycelium would not serve as a substitute for the mushroom sporophore, however, since it lacks the structure and texture of the fruiting body, and this is of significant importance in a food product.

Growth

Styer[5,6] and other workers,[7–10] have demonstrated that mushroom mycelium will grow in liquid media on a variety of carbohydrates supplemented with inorganic or simple organic nitrogen compounds and mineral salts. There are some species which also require thiamine.[11,12] Growth will occur with even a small concentration of nitrogen in the medium, but this is reflected in a correspondingly small protein content in the mycelium.[8] The protein content increases with increasing nitrogen in the liquid medium, but this does not reach a value higher than is found in the fruiting body under normal growing conditions—35 to 45 per cent computed on the basis of a protein containing 16 per cent nitrogen. If the value of 11·79 per cent nitrogen, determined by Fitzpatrick *et al.*[13] for purified mushroom protein is taken, the protein content is 1·36 times as great.

The influence of pH, temperature and other physical factors has been studied, as well as nutrients, but some basidiomycetes and ascomycetes still fail to grow, or grow very poorly, in culture media. On the other hand, others which grow well in culture fail to adapt to submerged aerated growth. Of those types which do grow submerged, the nature of the mycelium may be quite varied. Some mushroom mycelia grow in small or large pellets while others give a dispersed or milky type growth. Jennison has reported a very slimy growth when he cultured truffles by the submerged method.[14] While the type of agitation can influence the character

of the growth and the size of the pellets, the dispersed growth is morphologically distinct. This distinction is the result of the formation of fine mycelium in the liquid medium as distinct from the ordinary coarse mycelium. The fine mycelium fragments into small pieces which form the secondary spores reported by several workers.[2, 15, 16] Szuecs[17, 18] notes that fine solids such as $CaCO_3$, $CaSO_4$ or cereal particles in the medium, serve as a support for the mycelial nuclei in colony formation and help to increase yields.

With *Agaricus blazei* we were unable to obtain satisfactory yields of mycelium in submerged propagation until the organism spontaneously produced the secondary spores and the dispersed type of growth.[2] Considering the yields of mycelium, Sugihara and Humfeld[16] obtained as much as 60 grams of dried mycelium per 100 grams of sugar used in the liquid medium. Eddy,[12] on the other hand, obtained only half this yield even though he used a complex organic medium, whereas Sugihara and Humfeld had used a simple medium of sugar, urea, and mineral salts. The difference between the results of these workers appears to arise from the difference in the nature of the growth, as mentioned before. A study of Sugihara and Humfeld's data[16] indicates that the yields were usually higher when the mycelium had dispersed growth and secondary spores than when it occurred in the pellet form. In Eddy's experiments, the mycelium was apparently in the undispersed form, thus accounting for the lower yields. With *Morchella esculenta*, on the other hand, Szuecs[17] has reported 59 grams (dry weight) of pellet-like mycelium from 100 grams of sugar in the medium. In large-scale production-sized runs with *Morchella* at the Commercial Solvents plant at Terre Haute, Indiana, yields of mycelium of two pounds or more per gallon, containing approximately 90 per cent moisture (similar to the moisture content of normal mushroom fruiting bodies) were obtained.[19] A plant having 10 half-million-gallon fermentation tanks, such as those employed in the production of industrial alcohol from blackstrap molasses, with a yield of two pounds of mushroom mycelium per gallon, could produce in two weeks mushroom mycelium exceeding the quantity of mushroom fruiting bodies produced in the United States in a year.

Robinson and Davidson[20] have described the procedure employed in 100- and 2,000-gal vat runs for research purposes. For

preparation of the inoculum, the mycelium from an agar slant is homogenized in a blender, transferred to a 250-ml shake flask and shaken at 100 rev/min for 72 hours. With less agitation the mycelium grows together to form large aggregates while at excessive speeds nodules do not form well.[17] For the large-scale production, the inoculating material from the shake flask or an aerated culture bottle is put into a 5-gal container and is homogenized with a high-speed impeller. The impeller speed is then slowed after homogenization so that nodulation can take place. Nodules of the morel mycelium thus produced are from 0·25 to 1·0 in. in diameter. These are dewatered to 90 per cent moisture whereupon they can be canned, frozen, dried, powdered, or converted to a liquid, flavour concentrate. If the mycelium is dried, it should be dried at 110°F, for a higher temperature produces an inferior product. More efficient growth without contamination was obtained at temperatures below 70°F. During the run the pH decreases until almost all the sugar has been utilized, after which the pH begins to rise. The best time to harvest the crop has been reported as just before this pH rise.

Species

It is interesting to note that of 50 strains of *Agaricus campestris* cultured by Humfeld and Sugihara, only three became adapted to submerged propagation. The others grew only slowly with pellet formation.[8] These three strains were unable to establish themselves on compost which is used for mushroom production. Szuecs similarly failed to obtain survival of the submerged-grown mycelium when he sprayed it on the surface of fermented manure under non-sterile conditions.[4] These results would indicate that the dispersed mycelium is, in fact, a physiological mutation. In all their studies, Sugihara and Humfeld successfully grew 20 species of mushrooms in submerged culture.[16] All of the cultures were obtained from tissue of fresh sporophores. Of these species, the only one other than *A. campestris* which had a pleasant flavour was *Lepiota rachodes*.

Eddy studied 20 species, all but one of which were obtained from a culture collection. In addition, he studied seven strains of *A. campestris* which were obtained from commercial mushroom

growers. Of the different species, only *M. esculenta* and *Psalliota* (*Agaricus*) *campestris* grew well on synthetic media. *Coprinus comatus*, the only species he found to produce a strong mushroom-like odour, required a medium containing an appreciable quantity of dried autolyzed yeast. It would cost as much to produce the mycelium from this medium, however, as it does to produce mushroom powders now selling commercially. Robinson[19] has grown four species of *Morchella* in the laboratory: *M. esculenta*, *M. crassipes*, *M. angusticeps*, *M. hortensis*, and several strains of some of these, *M. crassipes* giving the best flavour. Benko[21] grew 64 species or strains of basidiomycetes in liquid culture and only five had any detectable mushroom flavour. This flavour was evident only in the most concentrated condition, namely the dried mycelium. Further, Benko grew cultures from 16 different samplings of commercial mushrooms (*A. campestris*) and found that only one of these produced the true mushroom flavour. In this case the flavour was fully evident in the fresh mycelium.

Flavour

In the past decade there have been several indications that commercial production was imminent, yet, up till now, it has not materialized. Mushroom mycelium is nutritious and its production presents no technological difficulties. But, as Cervantes put it in Don Quixote, 'the proof of the pudding is in the eating'. In this case, the eating demonstrates that the mycelium is mushroom-like in flavour, but not the equivalent of the tissue from the sporophore. There appears to be some confusion in the literature dealing with the flavour of the mushroom mycelium. This confusion is understandable since evaluation of flavour depends upon subjective reporting. It is complicated in the separation of odour and taste and in the difference between the odour of the fresh, uncooked mushroom or mycelium and that after it is cooked. The unique, earthy aroma of the uncooked sporophore is similar to that of the mycelium from a mushroom bed or from that produced in liquid culture. The taste and flavour after cooking, however, may be another matter. Cooking intensifies the flavour and may change it qualitatively as well. It is possible that the mycelium contains some of the flavour constituents of the fruiting

body but not all of them. In the writer's opinion, the mycelium at its present best, while similar in flavour to the sporophore, falls short of matching it in intensity and kind.

Eddy has introduced the sobering thought that even were the mycelium to achieve the full flavour of the *A. campestris* fruiting body, it would still fail to compete with the commercial mushroom powders imported into England from European countries, which are produced from wild mushrooms such as *Boletus edulis*. The powdered mushroom product which he produced from fresh cultivated mushroom fructifications lacked the strong flavour of the imported powder, and this finding is supported by commercial experience.[22]

There are indications that the type of growth has an influence on flavour. In our own work, we reported the lack of flavour in the dispersed-grown mycelium of *A. blazei*. Following our published report, we produced some of the original unmutated strain, which grew only very slowly in submerged culture, and this had an aroma which was instantly recognized as mushroom by everyone who smelled the flask. The mycelium of the morels, *M. esculenta* and *M. crassipes*, produced by Szuecs and by Robinson, showed pellet-type growth rather than dispersed growth. The mycelium of *A. campestris* of Sugihara and Humfeld, which was characterized by the dispersed growth, was reported to be 'nut-like, cheese-like, and pleasant' by a taste panel.[16]

O'Neill[23] found that NRRL strain 2334 of *A. campestris* grown in submerged culture was given a flavour score of 7·3 by a taste panel as compared with a slurry of fresh mushrooms, blended to a similar texture, which had a score 8·3. Of a taste panel of 19 persons, 15 found the mycelium to be an acceptable table item, whereas 4 disagreed. Those who became used to the taste of the mycelium found it more acceptable than those who were not familiar with its taste. It is interesting that Benko repeated the work of O'Neill but obtained no flavour with the same strain. Out of 80 different cultures which he investigated, Benko found one culture which permitted his conclusion that mushroom flavour can be produced in submerged aerated culture. This culture was obtained from commercial mushroom production of *Agaricus bispora* (*campestris*). It grew only slowly in submerged culture and did not sporulate but produced small pellets. When harvested

after 10 days on an organic medium, 9 out of 10 tasters stated that the mycelium, which was fried in butter, had a flavour as strong or stronger than that of fresh mushrooms similarly prepared.

A chemist for one of the large breweries working on the production of mushroom mycelium told the writer that the mycelium that was produced had a flavour that was considered highly acceptable by a taste panel but that the flavour was lost on canning, thus causing the company to abandon the project.

It is interesting to observe that mycelium grown on solid media or on the surface of shallow liquid media is reported to have flavour, whereas the same species grown submerged does not. Sugihara and Humfeld point out that mycelium grown in solid media possesses the typical mushroom odour but the odour is not detectable in mycelium grown in submerged culture. Szuecs was issued a patent [24] for the production of essence of mushroom by the growth of *Psalliota campestris* on a solid medium. When Eddy harvested the mycelium of *C. comatus* after three weeks growth on the surface of a shallow liquid medium, there was a strong, unmistakable, mushroom-like odour, yet mycelium of the same organism grown in an aerated fermentor had no odour. Humfeld and Sugihara [8] found that inorganic nutrients had an effect on flavour. They found that higher concentrations of inorganic salts (excepting magnesium) were required to bring out the maximum flavour than for obtaining merely the maximum yield of the mycelium. The effect of yield on the flavour has been noted by Robinson. While he was able to obtain a yield higher than two pounds per gallon, this increased yield of mycelium was found in production runs to result in reduced flavour. For optimum flavour, the yield was held to slightly less than two pounds per gallon.

Flavour Enhancement

Various methods have been suggested to enhance the flavour of the mushroom mycelium. Most are methods of cooking or autolysis. Robinson, for example, refers to the cooking technique as important in enhancing flavour. Block *et al.* reported that when the mycelium from the synthetic medium was gradually heated to about 75°C, the cells were autolyzed, as shown by the

decrease in viscosity of the mycelium paste. This treatment brought out the flavour, but unfortunately the flavour in no way resembled that of a freshly cooked mushroom.

A patent for flavour enhancement of mushroom mycelium[25] involves the addition of 4 per cent salt and the storage of the mycelium at 4°C for eight days. This process is said to be independent of oxygen but will not work if the mycelium has been previously sterilized, indicating that it is enzymatic in nature. Still another enhancement procedure is to continue the aeration of the submerged culture of the mycelium one or two days after all the sugar has been utilized, whereupon, it is said, the strong mushroom flavour is developed.[8] Eddy tried these methods with little success. Nevertheless, he came to the conclusion that the most probable explanation for the development of flavour after three weeks of static surface-culture (whereas no flavour was obtained in the submerged growth) was that the flavour is produced by autolytic changes in the aging mycelium. This conclusion was reached after first eliminating two other possibilities; namely that aeration in the fermentor was deficient compared with that of surface growth, and secondly that the flavour constituents were volatile and were swept away during the aeration of the medium. In the first case, he used pure oxygen and demonstrated that the aeration rate greatly exceeded the uptake of oxygen. But even with pure oxygen no flavour was obtained. In the second case, he used freezing traps to condense any volatile materials, but none were found that had the mushroom-like odour.

There is another possible enhancement procedure to which the writer once alluded facetiously. In experiencing the bland taste of the mushroom mycelium and the fact that the mycelium readily picks up flavours to which it is exposed,[2] he suggested that an easy solution might be to grow the mycelium on a medium of strongly-flavoured mushrooms. O'Neill tried this procedure, adding slurries or extracts of fresh mushrooms to the medium of Humfeld and found that with at least one strain there was an increased mushroom flavour. On the other hand, Benko[21] made an extensive study of natural extracts and other additives which included mushroom extracts, decayed wood, manure, soil, rice straw, alfalfa, grains, leaves, casein hydrolysates, monosodium glutamate, xylan, xylose, and giberellic acid, and obtained no

increase in flavour. No improvement in flavour was found by variation of temperature of growth, masceration of the mycelium, or by the addition of enzyme extracts from fresh mushrooms. It should be noted that Benko employed five weakly-flavoured cultures in these experiments rather than the one culture out of 80 which he found to have a strong mushroom flavour.

O'Neill irradiated his cultures with gamma rays from a cobalt-60 source and reported somewhat increased flavour from the submerged-grown mycelium. Szuecs[18] claims an intensely flavoured *A. campestris* mycelium when lecithin or an edible fat is added to a medium containing sugar, yeast extract and mineral salts.

Further Considerations

In view of the obvious potentialities of the process of the production of mushroom mycelium, a few thoughts on further channels of investigation might be desirable. The mushroom mycelium has been shown to be a highly nutritious product like the sporophore. There are no unusual technical difficulties holding back the production of such a food. The bottleneck at this point would appear to be in the flavour. If the flavour is the problem, it appears to be the point of departure for further investigation.

One question which must be answered definitely is whether the flavour constituents are a product of the specialized cells of the sporophore alone or whether they are also produced by the mycelium. One approach to this problem would be to devise a method for harvesting mycelium of mushroom fungi produced on solid media, such as used for commercial mushroom production. Conversely, it would be of interest to grow fruiting bodies from mycelium which has been produced in submerged culture. The determination of when and where in the sporophore the flavour constituents originate would be useful. The chemical identification of the flavour constituents would also be of underlying value to this whole problem. It would then permit a concrete and not merely a subjective approach. If the major constituents of the flavour are known, it might then be possible to obtain biological synthesis of the flavour ingredients by the mushroom mycelium with the use of chemical percursors in the medium.

Such methods, however, should not necessarily exclude a continuation of empirical experiments to supply the necessary nutrients and conditions for flavour synthesis. The problem beckons: its solution would represent a major achievement in food production.

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Population Dynamics in Suspension Cultures of an Animal Cell Strain*

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Summary. A composite of several studies by the authors is presented in an attempt to illustrate the use of the suspension culture method to analyze population dynamics of an animal cell strain. On the basis of these studies the nature of the lag and plateau phases is discussed. More extensive discussions of the various studies in relation to the work of others appear elsewhere.

Introduction

Since the demonstration of the suspension or submerged culture method by Owens, Gey and Gey,[1] and its development by Earle and associates,[2–4] a number of cell strains, both epithelial and fibroblastic, have been adapted to grow in this fashion.[5–11] While some of the strains grow as small aggregates of cells, a number grow as monodisperse suspensions when proper chemical and physical conditions are provided. Application of techniques well established in microbiology has permitted characterization of cell growth on a quantitative basis.[12–14]

This presentation will illustrate the way in which analysis of population behaviour has been applied, by us, to the study of suspension cultures of L-strain fibroblasts in an effort to characterize the nature of the cell growth cycle and thus to improve culture procedures. It is of considerable interest that these observations with tissue cells closely parallel the results obtained with bacterial and protozoan populations.[15–17]

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Materials and Methods

The cells used for this study were the L-strain 929 mouse fibroblasts.[18] Stock cultures were maintained in a protein-free medium composed of chemically defined medium 199 of Morgan, Morton and Parker,[19] supplemented with 0·5 per cent Bactopeptone as described by Waymouth.[20] An alternative medium for experimental studies has been Eagle's basal medium[21] supplemented with yeast extract, lactalbumin hydrolysate and peptone.[22]

For growth of these cells in suspension, it is necessary to add a macromolecular component to the medium to prevent clumping. In some of our studies, 5 per cent whole horse serum was used, but methylcellulose* was found to be more effective. It was added in a final concentration of 0·12 per cent. Growth is quite similar with either compound, though the generation time is somewhat longer in methylcellulose-containing cultures. Their mechanism of action appears to be chemical bonding through polyvalent cations to reactive sites such as free carboxyl groups on the cell surface. The cell surface thus becomes saturated and similar bonding between reactive groups on adjacent cells is prevented.[23]

Under these circumstances the cells were maintained as monodisperse suspensions throughout most of the growth cycle. Only after cell multiplication had ceased did cells begin to adhere to the walls of the culture vessels and to clump. To overcome these difficulties we added a dispersing agent, Darvan 2†, to the medium. This is a compound of a type widely used to disperse clays, dyes and other colloidal materials. It is a polymerized organic salt of a sulphonic acid. In aqueous solution it dissociates to give highly charged anions which coat the cells and impart their negative charge to them.[24] In a concentration of 0·05 per cent, Darvan 2 is non-toxic and keeps the cells dispersed through the entire growth interval. An additional use of this compound is in solutions used to dilute cells for counting.[22] Frequently cells which have been adequately dispersed will reaggregate in salt solutions.

The cultures used in the experiments reported here were prepared as follows: cells from a culture in late log or early plateau phase were seeded in growth medium at a concentration of 200–300

* Methocel 15 cps, Dow Chemical Co., Midland, Michigan.

† Darvan 2 ®, R. T. Vanderbilt Co., New York, N.Y.

thousand cells/ml. Approximately 100 ml of cell suspension was added to each of several 250-ml Erlenmeyer flasks. The pH was adjusted to approximately 7·4 and the flasks were tightly stoppered. The cultures were incubated on a rotary action shaker* with a 1-in. radial stroke at 100 rev/min. The temperature of incubation was 35°C. The cultures were not gassed.

As shown in Fig. 1, replicate cultures were used to obtain measurements of cell number, packed cell volume, cell size, protein

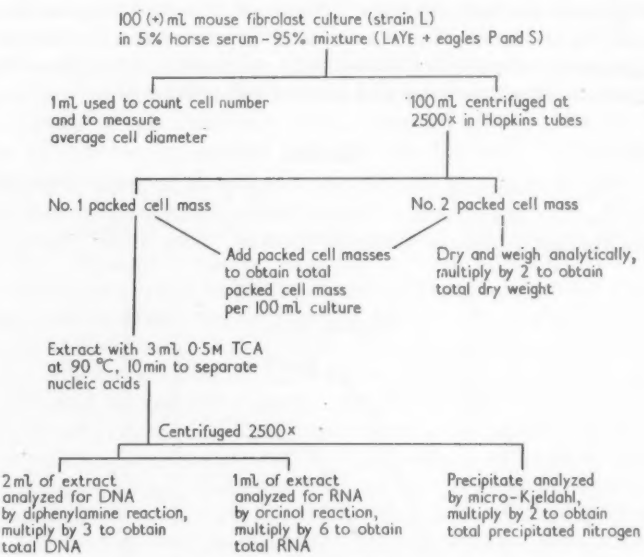
100 (+) ml mouse fibrolast culture (strain L)
in 5% horse serum – 95% mixture (LAYE + eagles P and S)
1 ml used to count cell number and to measure average cell diameter
100 ml centrifuged at 2500× in Hopkins tubes
No. 1 packed cell mass
No. 2 packed cell mass
Add packed cell masses to obtain total packed cell mass per 100 ml culture
Dry and weigh analytically, multiply by 2 to obtain total dry weight
Extract with 3 ml 0·5M TCA at 90 °C, 10 min to separate nucleic acids
Centrifuged 2500×
2 ml of extract analyzed for DNA by diphenylamine reaction, multiply by 3 to obtain total DNA
1 ml of extract analyzed for RNA by orcinol reaction, multiply by 6 to obtain total RNA
Precipitate analyzed by micro-Kjeldahl, multiply by 2 to obtain total precipitated nitrogen

Fig. 1. Analysis of the development of an animal cell population growing in suspension culture

nitrogen, ribonucleic acid, deoxyribonucleic acid and dry weight. Cell number was determined by counts in a haemocytometer or by use of an electronic cell counter.† The latter proved to be of great value as it reduced the counting error from a level of 15–20 per cent with the haemocytometer to approximately 1–2 per cent.

* Eberbach and Son Co., Ann Arbor, Michigan.

† Coulter Electronics, Chicago, Illinois.

Reduction in time required for counting was also an important factor. Cell size determinations were made either by measurement of diameter with a calibrated ocular micrometer or by direct measurement of volume with the electronic counter. Packed cell volume was measured using Hopkins vaccine tubes. DNA was determined by the diphenylamine reaction, RNA with the orcinol reaction and protein nitrogen by the micro-Kjeldahl method.[14]

In other experiments glucose utilization was followed by making serial determinations of the residual glucose in the medium using the glucose oxidase method. Lactate in the medium was determined by the method of Barker and Summerson. In the latter experiment mean cell volume was calculated from the determinations of cell number and packed cell volume.[25]

Results

Fig. 2 illustrates the nature of the growth cycle of strain L fibroblasts in suspension. The cultures were inoculated with cells from the early plateau phase of growth. The initial pH of the

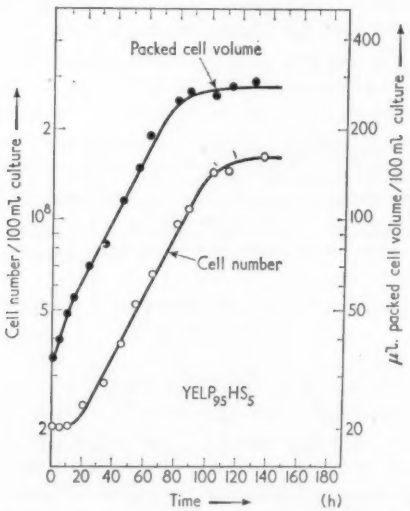

Fig. 2. Relationship of packed cell volume to the number of cells during the growth cycle of L-strain fibroblasts

medium was 7·4. It will be noted that a brief but well defined lag in cell number increase occurred which was followed by 3–4 days of logarithmic population increase. The population normally plateaued at approximately 1·5–2·0 million cells/ml. No medium changes were made during these experiments. The generation time, or the time required for the population to double, can be calculated readily from such a curve and in these experiments ranged from 28–30 h.

A comparison of the curve obtained by plotting packed cell volumes against time in hours with a similar plot of cell numbers indicates several points of interest. It will be noted in Fig. 2 that during approximately the first twenty hours there was a rapid increase in packed cell volume which was not associated with an increase in cell number. Also at the end of this interval the rate of increase in packed cell volume declined. Subsequent experiments indicated that the rate of increase may continue to decline slowly during the entire log phase and packed cell volume increase, therefore, does not exactly parallel the increase in cell number. Finally, it can be seen that increase in packed cell volume can cease as early as 18–20 h before cell number increase stops.

An analysis of cell size distribution during various phases of the growth cycle showed that there were major shifts in size distribution which correlated with the points of difference between cell number and packed cell volume. It will be noted in Fig. 3 that cells at zero time, which had been taken from an early plateau phase culture, were predominately small cells. After 24 h, when the log phase began, distribution of cell size was almost reversed. Within a short time after the culture had entered the log phase size distribution became random. Further studies have shown that the distribution remains random until near the end of the log phase though the mean cell volume may gradually decrease during much of this interval.

When a culture entered the plateau phase, as shown by a cessation of cell number increase, there was an accelerated decline in the mean cell volume which was reflected by an increase in percentage of small cells. At the end of the plateau phase, just before the onset of the phase of decline, the cells were uniformly small but also uniformly viable. Within a few hours, however, viability was completely lost and the cells degenerated rapidly.

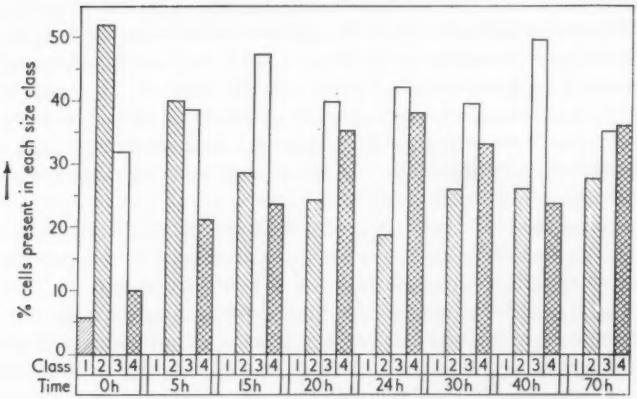

Fig. 3. Cell size distribution analyses at various times during the growth cycle of L-strain fibroblasts
Class 1: 1,150 μ^3 cell volume Class 3: 2,150 μ^3 cell volume
2: 1,500 μ^3 cell volume 4: 2,950 μ^3 cell volume

Thus it would appear that the lag phase represents a period of growth required to prepare the inoculated cells for division. That such a relationship does exist is further indicated by the fact that when care is taken to properly adjust pH and temperature the length of the lag phase for the L-strain is directly proportional to the age of the cells used for the inoculum. Thus cells in early logarithmic growth phase showed no lag when put into fresh medium. When inocula were obtained from progressively older cultures from late log to late plateau phases the length of the lag period increased.[26]

To clarify further the nature of this relationship, other parameters of population development were studied. Data presented in Fig. 4 suggest at least two mechanisms were involved in the cell size changes noted during the lag phase. In contrast to the changing rate of increase in packed cell volume, dry weight increased in a linear fashion suggesting that the early rapid rise in packed cell volume might be due in part to hydration of the cells. Support for this hypothesis was obtained from studies of glucose utilization.[25] The data in Fig. 5 show that the rapid

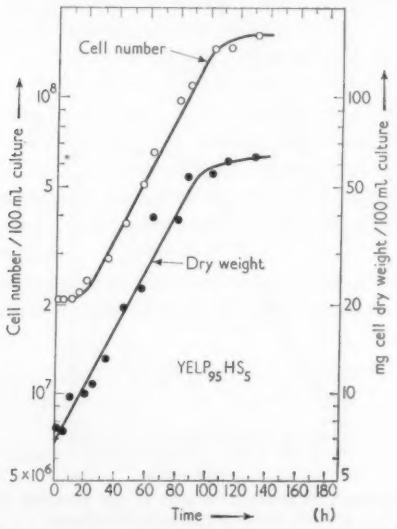
Cell number
Dry weight
YELP95HS5
Cell number / 100 mL culture
mg cell dry weight / 100 mL culture
Time (h)
0 20 40 60 80 100 120 140 160 180

Fig. 4. Relationship of cell dry weight to the number of cells during the growth cycle of L-strain fibroblasts

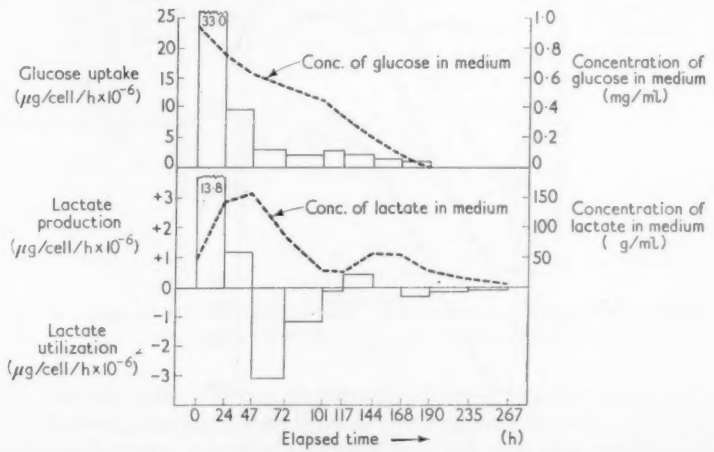
Glucose uptake (μg/cell/h×10⁻⁶)
Conc. of glucose in medium
Concentration of glucose in medium (mg/mL)
Lactate production (μg/cell/h×10⁻⁶)
Conc. of lactate in medium
Concentration of lactate in medium (g/mL)
Lactate utilization (μg/cell/h×10⁻⁶)
Elapsed time (h)
0 24 47 72 101 117 144 168 190 235 267

Fig. 5. Utilization of glucose and production of lactate during the growth cycle of L-strain fibroblasts

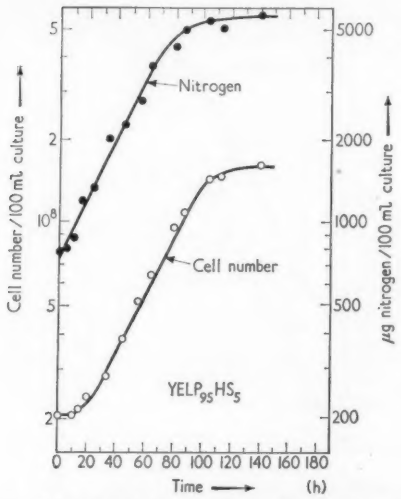

Fig. 6. The relationship of protein content to the number of cells during the growth cycle of L-strain fibroblasts

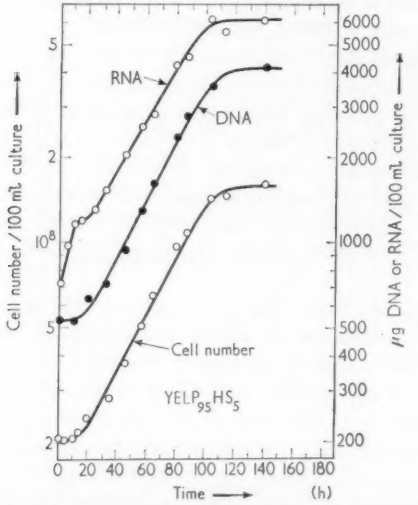

Fig. 7. The relationship of RNA and DNA content to the number of cells during the growth cycle of L-strain fibroblasts

increase in mean cell volume during the lag phase is correlated with rapid glucose uptake by the cells.

As seen in Fig. 6 protein N also increased in a linear fashion, thus confirming the results obtained by determination of dry weight. Increase in protein N stopped before cells entered the plateau phase. From Fig. 7 it can be seen that ribonucleic acid increased most rapidly during lag phase and then continued increasing at a steady, but slower, rate throughout most of the log phase. Deoxyribonucleic acid followed closely the pattern of cell number increase.

Fig. 8 illustrates some of the apparent relationships between cell number, cell size, cell protein content and carbohydrate utilization

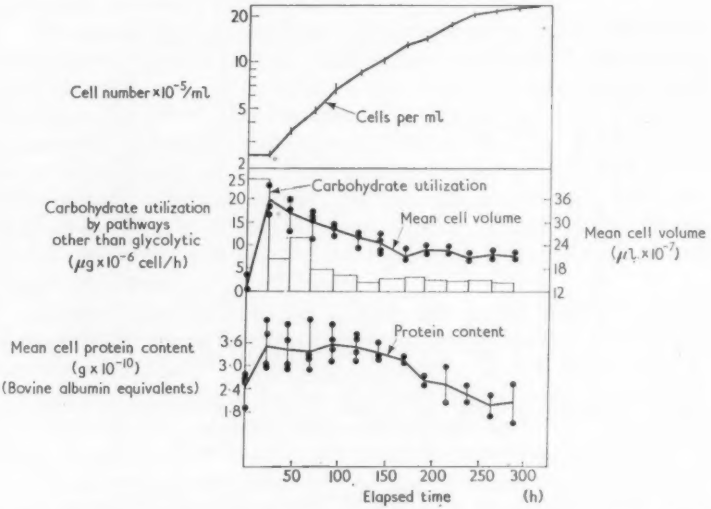

Fig. 8. Changes of mean cell volume, carbohydrate utilization and protein content during the growth cycle of L-strain fibroblasts

by L-strain cells. It can be seen that glucose uptake was most rapid during the lag phase, and, as has been mentioned, a parallel existed between increase in individual cell size and uptake of glucose. Moreover, the protein content per cell increased rapidly

during this interval. Mean cell volume decreased continuously after the end of the lag period and mean protein content decreased beyond the late log phase.

As seen in Fig. 9 glucose was completely exhausted early in the plateau phase. During the lag phase, when glucose was rapidly taken up, there was a rise in lactate in the medium. Beginning about the midpoint of the log phase this was reversed and lactic acid was removed from the medium until just before plateau was reached. At this time a secondary rise in lactate occurred and continued until the glucose was exhausted. Finally the remaining lactate was utilized. The changes in glucose uptake and in lactate production and utilization are quite dramatic when plotted on a per cell per hour basis.

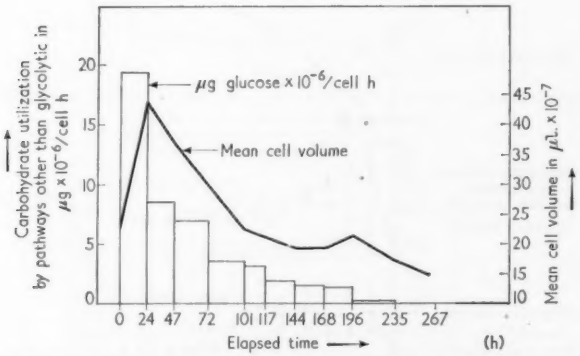
Carbohydrate utilization by pathways other than glycolytic in μg x 10⁻⁶/cell h
μg glucose x 10⁻⁶/cell h
Mean cell volume
Mean cell volume in μL x 10⁻⁷
0 24 47 72 101 117 144 168 196 235 267
Elapsed time
(h)

Fig. 9. Relationship of mean cell volume to carbohydrate utilization during the growth cycle of L-strain fibroblasts

Discussion

We believe the findings reported here to be in agreement with the hypothesis that the lag phase in L-strain cultures normally represents a period of substrate uptake and of synthesis of materials required to initiate cell division. It occurs when the population is not randomly distributed with relation to cell size and hence with respect to the amount of synthesis required for cell division. The smallest cells observed, which ranged in volume

from 1,200 to 1,500 μ^3, probably represent cells which have just divided and which must double their size before the next division. The largest cells, ranging from about 2,600 to 3,000 μ^3, would represent cells which have completed the synthesis needed for cell division. Variation in the ratios of different sized cells in an inoculum would thus be reflected in the length of time required to reach a random distribution characteristic of the log phase.

Further evidence supporting this hypothesis is the observation that cells taken from a culture in the late plateau phase, when used to initiate growth, increase uniformly in size and then divide synchronously.[26] Since such cells are almost entirely of the smallest size the duration of the lag period, which is about 20 h, should reflect the minimum time required for a cell to carry out synthesis necessary for division.

The reason for the continued decrease in mean cell volume and particularly for the gradual shift to uniformly small cells during plateau appears to be twofold. First, it will be remembered that increases in cell number occurred after there was a cessation of increase in dry weight and of protein nitrogen. This would result as a consequence of the random distribution of cell size if we assume that a portion of the cells had already reached maturity and were capable of dividing without further synthesis. Since these cells could not grow further a skewed distribution of cell sizes would result.

A second factor is related to the observation that glucose is exhausted as the cells enter plateau and that lactate is used up shortly after that.[25] Assuming glucose to be the major energy source, the cells would be forced to use endogenous metabolism for survival beyond this point. The rapid shift from high viability to no viable cells and subsequently degeneration at the end of the plateau phase suggests that such endogenous metabolism may continue until the structural and functional units of the cell are reduced to a point which is incompatible with cell integrity. Support for this supposition comes from the observation that the plateau phase can be extended from the normal duration of 3–4 days to as much as 2–3 weeks by a single addition of glucose at the onset of plateau.[27] No further increase in cell number occurs under these conditions but viability remains high throughout the prolonged plateau.

A practical application of the information obtained in these studies has been the construction and operation of a continuous cell generator. This apparatus, which is shown in Fig. 10, maintains a constant cell population and a constant chemical environment by changing the medium completely during one generation time. Cells are removed with the used medium. The L strain has been maintained in this apparatus in continuous logarithmic growth for intervals of up to 35 days. The population level was maintained between 400 and 600 thousand cells per ml. The minimum generation time obtained was about 50 h. Medium flow rate was approximately 3–4 ml/h. No attempt was made to gas the cultures.

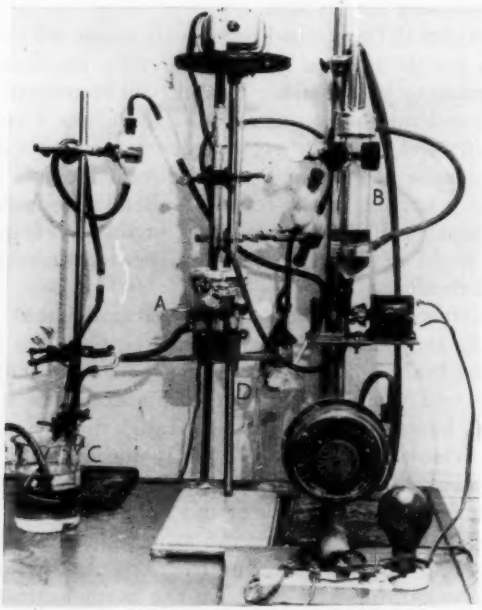

Fig. 10. Continuous cell generator for cultivation of animal cells
A generation vessel
B medium reservoir
C collection flask
D gas reservoir for equilibration

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Studies and Observations on the Growth of Mammalian Cells in Agitated Fluid Medium

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Summary. Fourteen mammalian cell strains were studied in the 'stirrer culture'. Fair to excellent growth was obtained with twelve of these strains while two strains failed to show evidence of proliferation. Growth curves, maximal population densities, and recovery following subculture of various cell strains, cultivated in this system, were described. Factors effecting the growth of cells under these conditions were outlined. Growth of cells in methods other than the 'stirrer culture' were reported. The practical importance of fluid suspension culture of mammalian cells, the potentialities and the problems involved in scaling such cultures to industrial size were discussed.

Introduction

The propagation of mammalian tissue cells in agitated fluid medium has become routine, or the object of research in many laboratories utilizing cultured cells. Although Owens and Gey[1] first described the growth of cells in fluid suspension, the current interest in this method of cell culture was stimulated by the reports of Earle *et al.*[2,3] who first described the growth of cells in rapidly revolving roller tubes and later, on a larger scale, in flasks agitated on a New Brunswick rotary shaker. In order to prepare an inoculum for a one or two litre shaker culture flask it was necessary to harvest cells from several conventional bottle cultures so that one could obtain a sufficient number of cells to initiate growth in this larger fluid suspension culture. In order to have a more convenient source of cells for this inoculum we developed the 'stirrer culture' as reported at the 1956 meeting of the Tissue Culture Association.[4] This culture consisted of a three-necked round bottom flask with a magnetic stirring bar suspended from the centre port by means of a nichrome wire. Later, a similar method was reported by McLimans[5] and, more recently,

Wallace and Cox [6] have described a method in which the magnetic stirring bar was not suspended.

Further modifications of, and data obtained from, our work with the 'stirrer culture' were reported in 1958.[7] Since this type of agitated culture has become quite popular the present paper was prepared to provide more details of its operation and to describe the results which have been obtained through its use in our laboratories. In addition, our experiences with other methods of fluid suspension culture will be described.

Materials and Methods

Culture Vessels

Three types of culture vessels were used in these studies and are described as follows.

The basic unit of the stirrer culture was a three-necked round bottom Morton type boiling flask (Fig. 1). The side necks were

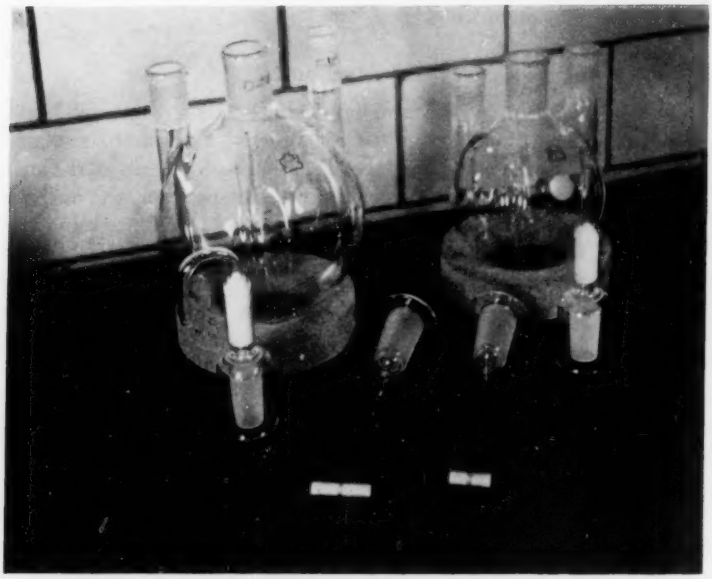


Fig. 1. Stirrer flask assembly (1 and 2 litre)

closed with ground glass stoppers fitted with a glass tube to permit gassing of the culture, and a cotton air filter was included. The centre neck was closed with a solid ground glass stopper to which a glass ring was sealed. A F3NS 'Sadie-Sampo' swivel* was attached to the glass ring and a 24 gauge nichrome wire with a Teflon coated magnetic stirring bar was fastened to the swivel. The wire was of such a length as to hold the stirring bar one-fourth to one-half inch above the floor of the flask. The flask was secured to a ring-stand and held about one inch above the magnetic base to reduce the heat transfer from the magnet. Flasks from 500 ml to 2 l. capacity were employed. A later modification of this equipment was made by indenting the sides of the flasks. Another type of stirrer culture flask (Fig. 2) was made in the form of a

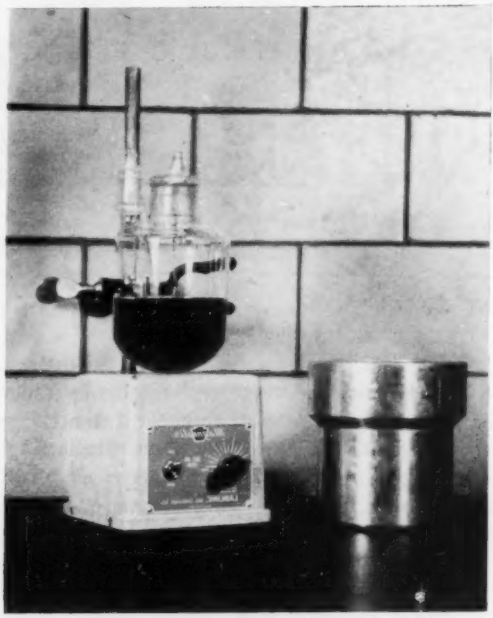


Fig. 2. Centrifuge stirrer flask

* Sampo Swivel Company, Redwood City, California.

centrifuge bottle to fit the No. 353A cup of the PR-2 International centrifuge. This flask was fitted with two ports in the top, one in the centre and one on the side. Three indentations were made in the sides and the centre port was closed with a ground glass stopper to which the stirring assembly was attached. This closure, as well as the side port, was used for gassing and removal of samples. Recently a new type of stirrer flask (Fig. 3) was

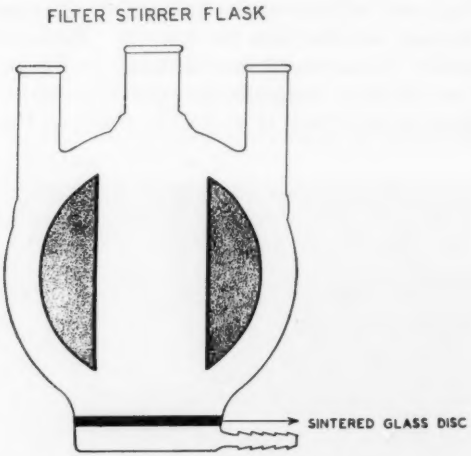


Fig. 3. Filter bottom stirrer flask

developed which permitted the direct removal of spent medium, thus eliminating the need to remove and centrifuge the cells. This culture vessel was fabricated from a stirrer flask to the bottom of which a sintered glass filter disc and reservoir were sealed. The New Brunswick fermentor used in our study was essentially the same as that described by McLimans[8] except that 7-l. jars and a stirring speed of 100 to 150 rev/min were used. The ports on the lid of the jar were closed with short pieces of clamped-off anode rubber tubing. The 'roller bottle' culture (Fig. 4) consisted of a 6-l. Pyrex bottle which was rotated on a U. S. Stoneware Corporation fixed-speed ball mill. The 6-l. bottle was

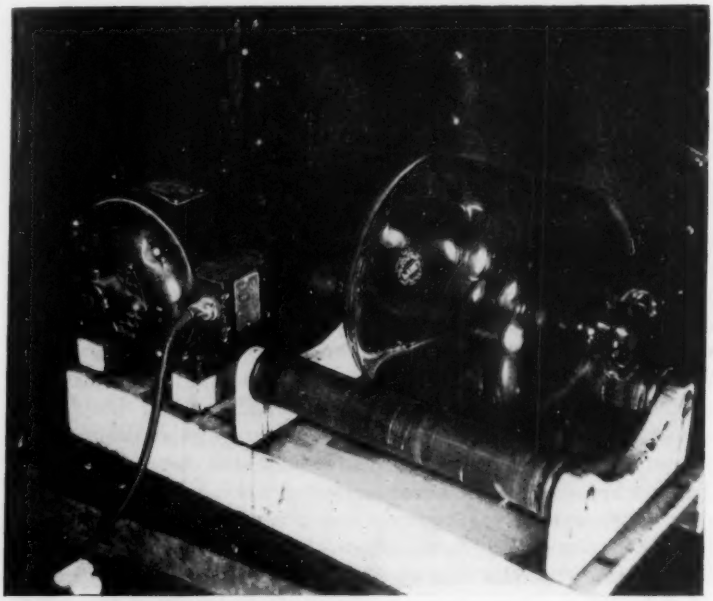


Fig. 4. Roller bottle and roller mill

encased in a metal sheath to increase its diameter, and thereby reduced its speed of rotation.

Cell strains. Cell strains used in these studies included strain L (NCTC 929), human amnion (AV_2, Rivadeneira), cynomolgus heart strain (CH, Salk), J96II (Osgood), HeLa (Gey), human skin strain (NCTC 2414, Perry), HEP_2 (Moore), Detroit 6 (Berman and Stulberg) and Lilly Laboratory cultures, LLC–M_1 originally explanted from a mouse lymph node, LLC–H_1 from human embryonic torso, LLC–MK_1 and –MK_2 from monkey kidney, LLC–WRC_{256}, the Walker rat carcinoma, and LLC–MS_{180}, mouse sarcoma 180. All strains were obtained from the originator as indicated, except HeLa, which was obtained from a commercial source and the human amnion which was made available by Dr. T. T. Puck.

Medium. In our previous report the medium used consisted of Earle's balanced salt solution containing chick embryo extract and horse serum. This was replaced with medium 199 containing 5 per cent horse serum. The medium 199 was made up with 1·68 g of $NaHCO_3$/litre and 100 units of penicillin and 100 μg of streptomycin/ml, and in some instances, the glucose concentration was increased from 100 mg/100 ml to 300 mg/100 ml.

Methods. The inoculum for fluid culture was generally prepared by removing cells from conventional monolayer cultures either by trypsin digestion, jetting or shaking the cells loose unless passage from one fluid culture to another was being made. In each instance, a portion of the old medium was retained and added with the cell suspension to an appropriate volume of fresh medium. An endeavour was made to obtain an initial cell concentration of 200,000 to 500,000 cells/ml. During the earlier stages of cultivation additions of medium were generally made as the population increased in order to build up the size of the culture. Later, when the culture was multiplying rapidly in the logarithmic growth phase, it was necessary to make complete medium changes. This was accomplished by aseptically removing the fluid and sedimenting the cells through centrifugation at 1,000 rev/min for 15 min. This operation was facilitated both by use of the centrifuge stirrer flask where it was not necessary to remove the fluid and cells from the culture flask in order to sediment for fluid change and also by the filter bottom flask which permitted constant removal of spent medium. Following sedimentation of the cells, the supernatant fluid was removed and the cells were resuspended in fresh medium. For gassing cultures, 5 per cent CO_2 in air was allowed to flow into the culture flask, through one of the ports designed for this purpose, for 5 min intervals once each 24-h period. Population levels were determined by the nuclei enumeration method of Sanford *et al.*[9]

In all instances the cultures were incubated in a 36°C walk-in incubator room except for the New Brunswick fermentors which were held in a 36·5°C water bath.

Results

To date, over 200 stirrer cultures have been initiated with 14 different cell strains. Fair to excellent growth was obtained with

12 of these strains in comparison with their growth in conventional flasks. Excellent growth was obtained with strains L, LLC–M_1, LLC–H_1, AV_2, HeLa, NCTC 2414 and J96II. Results of experiments with LLC–WRC_{256} and MS_{180} were erratic and less satisfactory while two strains, LLC–MK_1 and LLC–MK_2, failed to show evidence of proliferation under all experimental conditions. Preliminary results with Salk's cynomolgus heart strain were erratic due mainly to extensive clumping of the cells. During this phase of the study, cells for the inoculum were prepared by trypsinizing stationary bottle cultures. When trypsinization of the CH strain was replaced by jet transfer, markedly better results were obtained. Preliminary work with the Detroit 6 and HEP–2 cell strains indicated that these strains could be cultivated in the stirrer flask but appeared to be hampered in the same manner as trypsinized cynomolgus heart cells. Attempts to improve the cultivation of Detroit 6 and HEP–2 cells are under study. The mouse cell strain, LLC–M_1, which is used as a standard in our laboratory, was the first cell strain studied in the stirrer culture. Results obtained with it typify the type of growth which was obtained in this system of cell cultivation. To illustrate (Fig. 5), a culture was initiated with 76 million cells in 200 ml of medium. Following a lag phase the culture entered the logarithmic phase and remained in this phase until the culture was terminated after 15 days' incubation. At this time, $2{\cdot}7 \times 10^9$ cells were present in the culture.

The recovery of a cell population in a stirrer culture of LLC–M_1 cells following removal of approximately one-half of the cells from the culture is shown in Fig. 6. As seen, the population returned to about the same level within 48 h and if cells were not again removed the population continued to increase further, in this example, to a maximum of 2×10^9 cells. At this point, approximately two-thirds of the total cell population was removed and again a rapid recovery was noted up to the time the experiment was terminated.

Several human cell strains were successfully grown in the stirrer culture among which was the AV_2 human amnion strain. A typical pattern of growth of this strain is shown in Fig. 7. The culture was started in a 1-l. flask and on the thirteenth day a subculture was made to a second 1-l. flask and following an additional

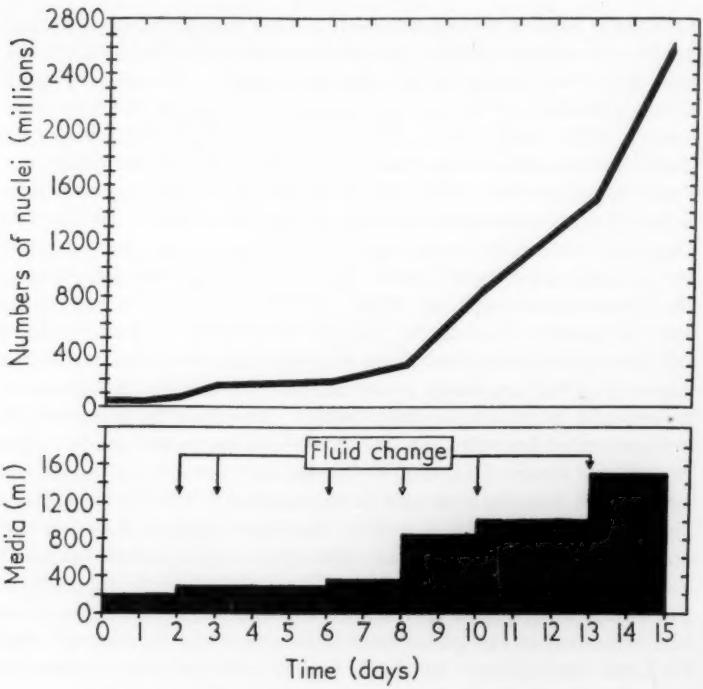


Fig. 5. Growth curve of LLC–M_1 cells in stirrer flask culture

13 day period another subculture was made to a 2-l. flask. In the early stages of growth in the 2-l. flask, removal of a small portion of the cells was not effective in stimulating cell proliferation. In the latter stages of growth, removal of 50–60 per cent of the total population was followed by complete recovery.

As mentioned previously, the cynomolgus heart strain was one which gave only fair results when the inoculum was prepared by trypsinization of conventional bottle cultures. The slow and erratic growth obtained over a 53-day period is shown in Fig. 8. The improvement made in the fluid cultivation of this strain by elimination of the trypsin treatment is seen in Fig. 9. This figure also shows the recovery of the culture after cell removal and of the

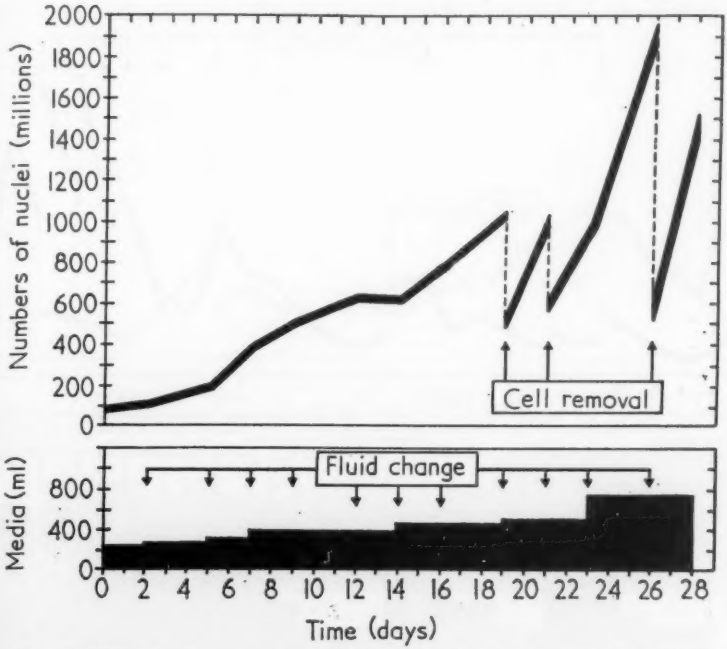


Fig. 6. Effect of cell removal on growth of LLC–M_1 cells in stirrer flask culture

growth of a portion of cells subcultured to a larger flask. In the original culture a total of $1{\cdot}3 \times 10^9$ cells were harvested during the 17-day period in which cell removal was accomplished. Four hundred and sixty million cells from the first removal were subcultured to a 2-l. flask. During the 17-day observation of this culture, the population increased to a total of $1{\cdot}4 \times 10^9$ cells.

Several factors were found to be important in initiating and maintaining stirrer cultures. Among these were inocula size and cell population/ml density during active growth phases. Inocula ranging from 50,000 to 1,000,000 cells/ml were employed; however, regardless of the cell type under study, approximately 400,000 cells/ml were found to be the minimal level from which the culture advanced to the logarithmic growth phase in a reasonable period of time. Inocula levels below 400,000 cells/ml given

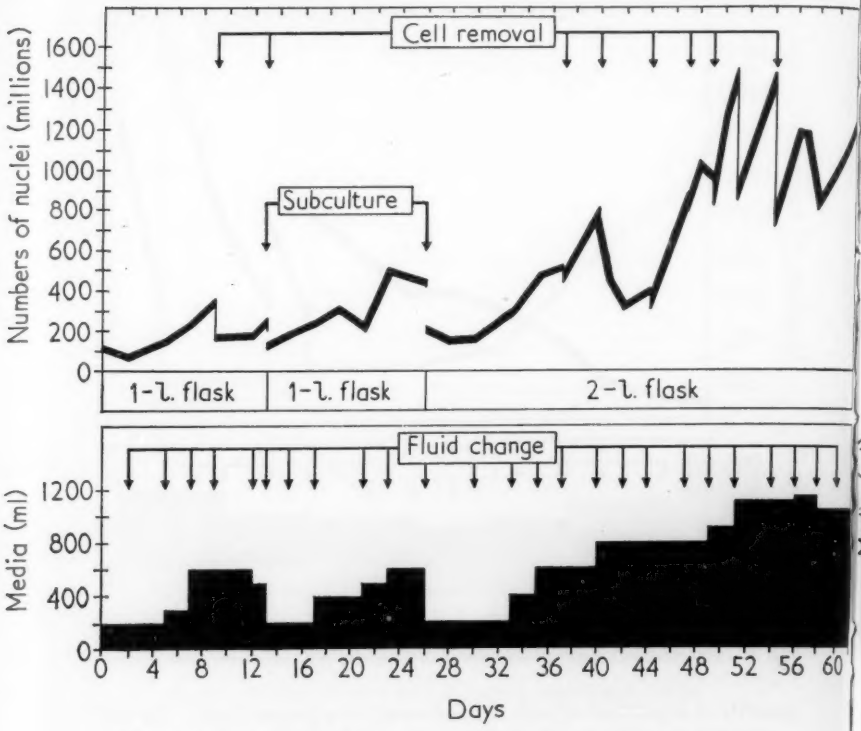


Fig. 7. Growth curve of human amnion (AV_2) cells in stirrer flask culture

sufficient time eventually reached the logarithmic growth phase but the time and medium expenditures through fluid change made this an inefficient operation.

Gassing of stirrer cultures was found necessary only when the medium levels reduced the airspace in a given flask to the point where oxygen supply and pH control were affected. Gassing was routinely performed at medium levels above 400 ml in the 1-l. flask and above 1,200 ml in the 2-l. flask. When medium levels were greater than indicated, failure to routinely gas the culture resulted in deterioration.

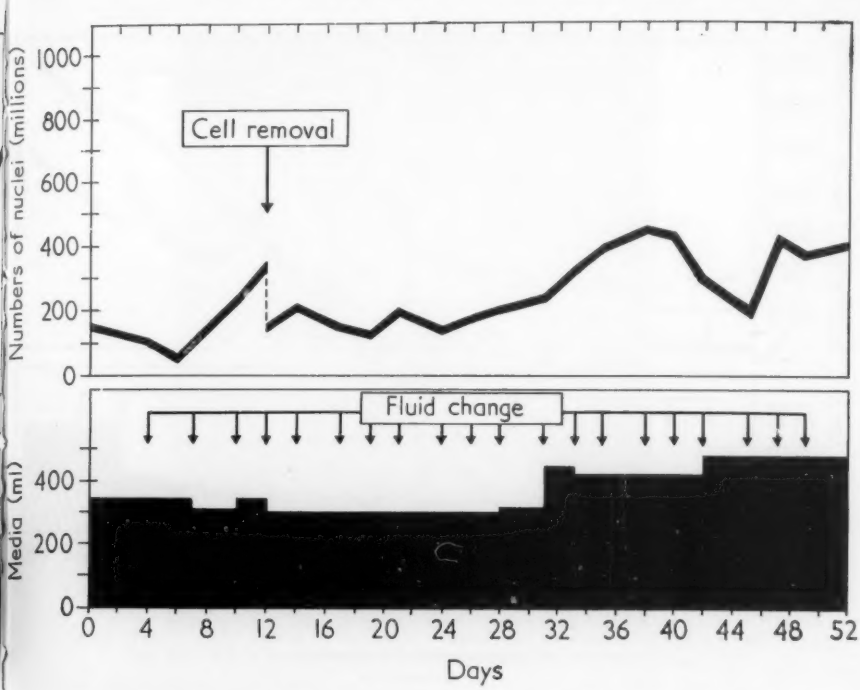


Fig. 8. Growth curve of cynomolgus heart cells in stirrer flask culture—trypsin treated cell inoculum

When smooth or symmetrically-shaped flasks were used, the cells had a tendency to collect on the glass and proliferate at the medium–air interface, thus complicating the quantitation of the cell population. Nuclei counts were subject to great variation in day to day counts due to the removal of cells from the fluid suspension, or by the cells growing on the glass sloughing off into the fluid phase. Thus, although growth continued in this type of flask, it was of an unsatisfactory type for research studies on cell populations and was not a true fluid culture since some of the cells were growing on the wall of the flask. To overcome this difficulty, four evenly spaced vertical indentations of the stirrer flasks were added to increase the agitation and thus eliminate clumping and

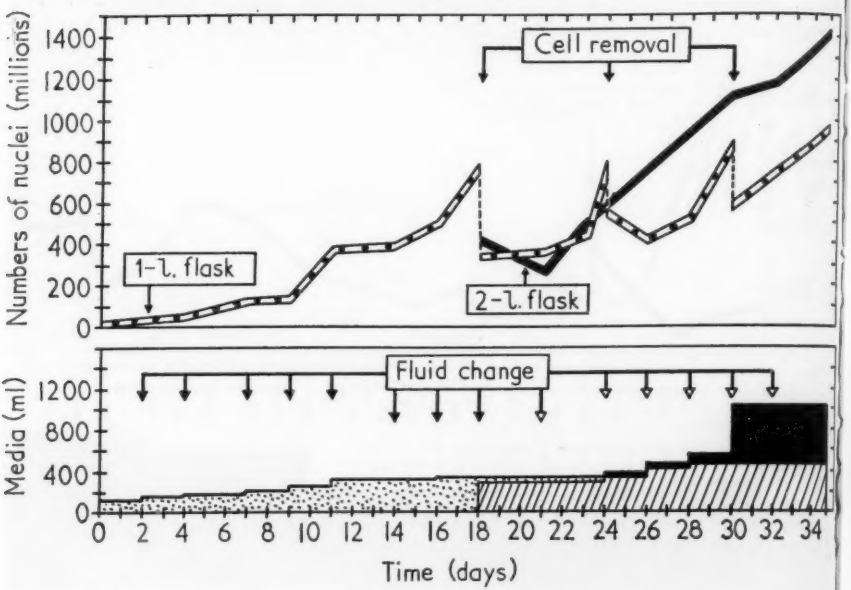


Fig. 9. Growth curve of cynomolgus heart cells in stirrer flask culture—non-trypsin treated cell inoculum

attachment of cells to the glass at the medium–air interface. Such indentations provided a counter-flow action to the horizontally rotated cells and successfully eliminated the build-up of cells on the glass.

Early in these studies a short lag phase was observed following removal of spent medium and the replacement with new medium that was stored at refrigerator temperatures. In conventional stationary flasks, the shallow layers of medium quickly warmed to the 36°C incubator temperature, but in the deep medium levels in fluid culture, time periods of two to six hours were necessary to raise medium temperatures to 36°C. Elimination of this short lag period was accomplished by the simple expedient of pre-warming the new medium to 36°C.

One of the greatest problems in the fluid cultivation of cells by these and other procedures arose from the need to periodically

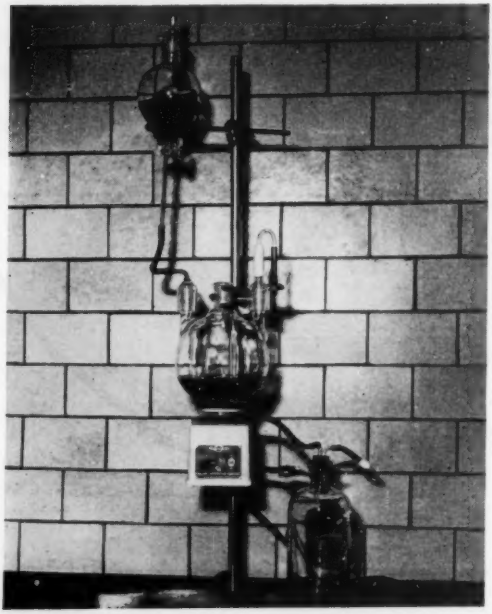


Fig. 10. Filter bottom stirrer flask assembled for continuous cell cultivation

withdraw and replace the medium in which the cells were suspended. One approach (Fig. 10) towards solving this difficulty was made through the design of a stirrer flask with a porous bottom which allowed the escape of medium but which retained the cells. Many attempts were made to utilize such a procedure in which either a slight vacuum or pressure filtration was employed, but in every instance, the filter, regardless of the type or porosity, quickly became clogged with cells. These difficulties were overcome in the filter bottom culture flask through the action of the stirring bar keeping the cells in suspension and off the filter surface, thereby permitting the gradual seepage of medium through the filter by the force of gravity alone. This type of flask has been used to accomplish periodic changes of medium, or, by the addition of a reservoir of medium connected to the top of the flask, a continuous

interchange of medium was possible. Cell strains LLC–M_1 and CH were successfully grown in the filter bottom flask and the results of these and other experiments will be reported at a later date.

Initial studies with the 7-l. New Brunswick fermentor were done with our standard cell strain, LLC–M_1. Fig. 11 illustrates

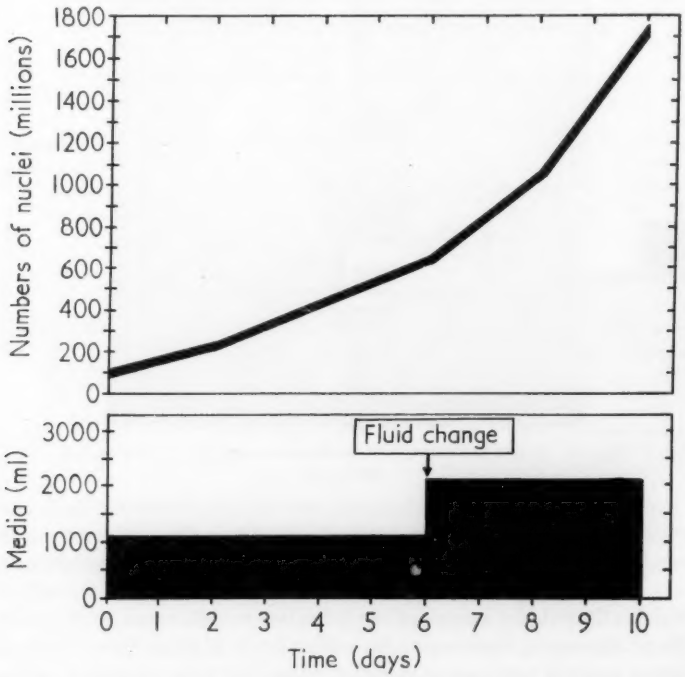
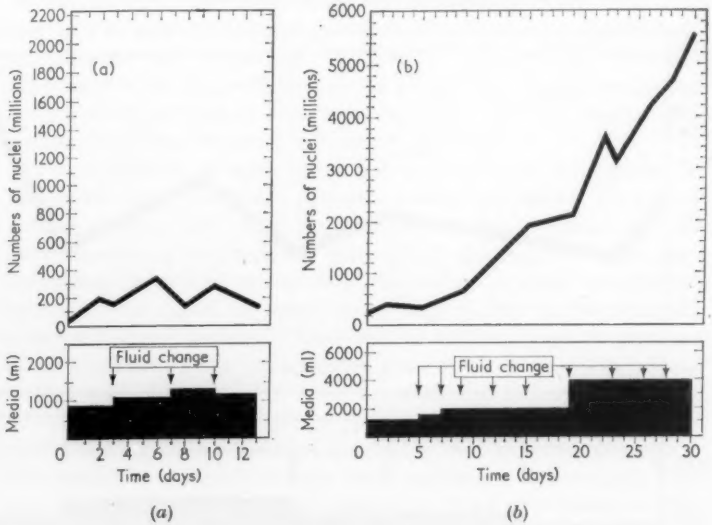


Fig. 11. Growth curve of LLC–M_1 cells in New Brunswick fermentor

the growth of this strain during the ten day incubation period. The culture was started with 109 million cells in 1,100 ml of medium. Although the concentration of 93,000 cells/ml was considered to be sub-optimal, growth was not adversely affected and a total cell volume of $1{\cdot}7 \times 10^9$ cells was achieved on the tenth and final day of this experiment. In the New Brunswick fermentor,

growth of trypsin-transferred CH cells followed the same pattern as in the stirrer culture, as observed in Fig. 12*a*. Inocula of CH cells prepared by jet transfer of stationary flasks or from logarithmic phase stirrer cultures, however, were readily cultivated in the fermentor as seen in Fig. 12*b*. In the latter experiment a total cell population of $5{\cdot}6 \times 10^9$ cells was obtained.



(*a*) (*b*)

Fig. 12*a*. Growth curve of cynomolgus heart cells in New Brunswick fermentor —trypsin treated cell inoculum

Fig. 12*b*. Growth curve of cynomolgus heart cells in New Brunswick fermentor —non-trypsin treated cell inoculum

As in the case of the stirrer culture, loss of cells from centrifugation and handling procedures was occasionally observed in the fermentor. Rings of cells at the air–medium interface, however, were not observed in the fermentor flasks even though the walls of the flask were symmetrical. This effect was considered to be due to the increased vertical turbulence set up by the propeller as opposed to the more lateral turbulence created by the blunt sided elongated bar used in the stirrer cultures.

In another approach to larger fluid cultures an investigation of growth of the LLC–M_1 cell strain was attempted in a 6-l. Pyrex bottle rotated on a ball mill apparatus. Cell survival was not obtained at bottle rotation speeds of 59 rev/min; however, cell survival and sporadic proliferation were obtained at 45 rev/min as observed in Fig. 13. Attempts to determine the factors necessary to increase the proliferation rate are under study.

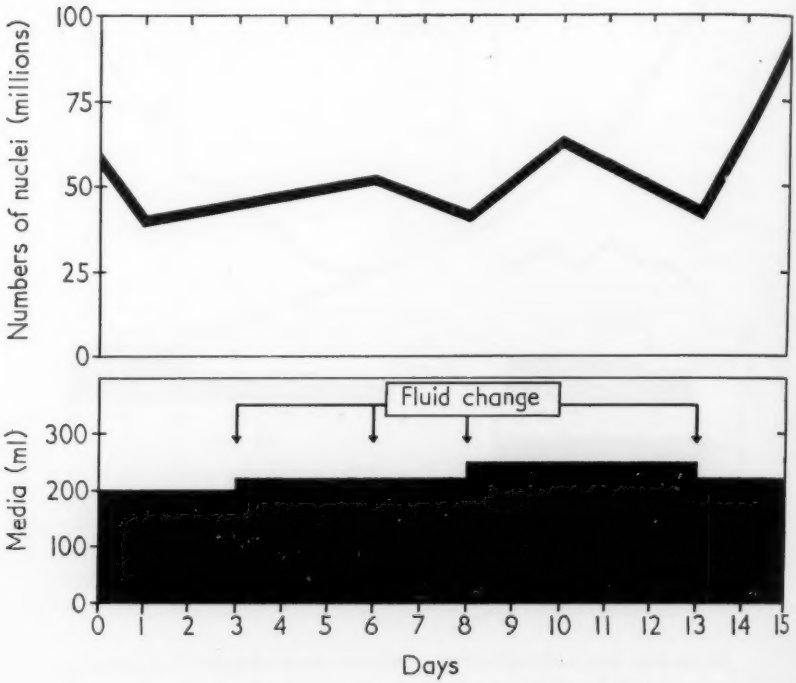


Fig. 13. Growth curve of LLC–M_1 cells in roller bottle culture

Discussion

The fluid cultivation of cells as described in this report was done under experimental conditions designed to study and to improve the methods. Therefore, long term or continuous use of the

stirrer cultures as a source of cells was not described. The method has been used successfully for this purpose, however, as mentioned in the report by Johnson.[10] Cultures can be set up and held in the logarithmic growth phase over extended periods by periodic removal of cells and replacement of spent medium.

The methods described, especially those involving the various modifications of the stirrer flask, can be used easily and economically in the research laboratory or elsewhere where industrial size cultures are not necessary. Several 1- or 2-l. stirrer cultures, stabilized in the logarithmic growth phase, can supply the inocula for hundreds of tube or flask cultures to be used for virus or drug assays and other research or routine problems.

The expansion of any of the methods of fluid culture to the industrial scale, however, presents several problems. Foremost among these is the need for periodic medium change and the development of methods to accomplish this on the large scale operation, or to find means of eliminating the need to replenish medium. Of equal or even more importance is the need to increase the cell density. Two million cells/ml were determined to be optimal in the logarithmic growth phase in most of our studies and this has also been reported by others. Graff and McCarty[11] reported somewhat higher cell densities in their cultivation of cells in the 'cytogenerator' but whether this equipment could be adapted to large scale cultures is not certain. We have on occasion obtained population densities of 3·5 million cells/ml and Earle[12] has reported in one instance densities as high as 30 million/ml with the mouse liver strain NCTC 1469. The actual factors governing the population densities are not clearly understood at this time but it is hoped that through further study more of these can be elucidated. It appears that with increase in cell density the need for rigid control of the culture as to gas, pH and medium, and perhaps other unknown factors, becomes more important. The nutritional requirements of cells also presents a problem in large scale fluid cultivation. With the exception of the growth of strain L in medium NCTC 109 plus methylcellulose by Bryant *et al.*[13] and in medium 199 with Bactopeptone by Merchant,[14] it has not been possible to grow cells in fluid suspension without animal serum included in the medium. Furthermore, in most instances higher serum concentrations were needed

than in conventional flask cultures of the same cell strain. A cheaper and more available source of suitable nutrients would be necessary for a commercial operation involving the fluid cultivation of cells on a scale approaching that of mould fermentation in the antibiotic industry.

Should these and other problems be overcome and the mass cultivation of mammalian cells become possible, it is not entirely clear at this time for what purpose these techniques might be employed. In the early days of penicillin production there was a need for mass culture techniques and these had to be developed quickly in order to provide the huge wartime demand for the new antibiotic. In the present case, however, attempt is being made to develop a mass culture method in advance of a specific need. There is, of course, nothing wrong with such an objective. There are, however, some foreseeable purposes for the large scale production of tissue cells. Their utilization in the area of virus vaccine production seems obvious if the enigma against the use of cell strains for this purpose can be overcome through further research. There is also the possibility of obtaining biologically or pharmacologically active substances such as hormones from such cultures, but this involves the further problems of finding suitable methods for the cultivation of highly specialized glandular tissues or cells. This has been difficult to do even in small slide or tube cultures.

It is obvious that the methods of fluid cultivation of animal cells which we and others on the symposium have, or will describe, work satisfactorily on a comparatively small scale and provide adequate tools for further study of the factors affecting the growth of cells in suspension. It is hoped that a continued effort to learn these factors will be made and that through such effort it will be possible in the future to grow or produce cells on whatever scale the need demands.

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Submerged Growth of Cells of Higher Plants*

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Summary. The submerged propagation of plant tissue and cell cultures has now reached the stage where mass amounts of material can be produced. In discussing these advances, various aspects of the technology involved are discussed: the methods of isolation and establishment of cultures, the media used for culture and their effects on growth, the systems used for growth and a comparison of their effectiveness.

Biochemical differences are shown between cultures grown under *in vitro* conditions and the organ from which they were derived. These differences are found among amino acids, organic acids, nucleic acids, and sugars. The potential use of the tissue culture approach for the study of the biosynthesis of 'secondary compounds' such as alkaloids and steroids is discussed.

Introduction

The early work of Gautheret,[2] White,[16] and Nobécourt[10] showed that the tissues and organs of plants can be grown *in vitro* under aseptic conditions. The outcome of these investigations was the application of plant tissue culture techniques to many areas of plant research, such as embryo culture, experiments on plant tumours, and nutritional studies. Recent developments have contributed information about two important aspects of plant cell growth: one was the isolation of clones of plant cells by Muir, Hildebrandt and Riker.[7] The other was the successful culture of plant cells in agitated liquid cultures. The latter was done by Caplin and Steward,[1] who grew carrot root explants in rotating liquid culture; Muir and Hildebrandt,[6] who experimented with the factors affecting the growth rates of tissues in liquid shake cultures, and Nickell,[8] who showed the feasibility of growing plant cells as micro-organisms. Important work is reported by Melchers and Bergmann,[5] who continuously cultured snapdragon cells in liquid media and also improved methods for aeration, sampling and volume control.

* Presented at the Symposium on 'Mass Propagation of Cells', sponsored by the Fermentation Subdivision, Division of Agricultural and Food Chemistry, 136th Meeting of the American Chemical Society, Atlantic City, N.J., September 15, 1959.

Isolation of Cultures

There are several methods which have been used for the isolation of plant tissue for culture *in vitro*.[3] The most important objective of these methods is to obtain aseptic cultures capable of being serially transferred for indefinite periods of time. The pathological tissues, such as crown galls (bacteria-induced tumours), virus tumours and genetic tumours, have been the subject of much pioneering work in the field of plant tissue culture. These pathological tissues synthesize many of the factors required for their growth including plant hormones, consequently the media on which they grow is simpler. The use of normal tissues requires the addition of plant hormones and other growth factors in order to maintain a state of proliferation. These normal tissues are perhaps the easiest to isolate and establish since one can start with aseptic tissue and thereby eliminate some of the difficulties in beginning a culture. A culture is started by sterilizing a number of seeds of the plant desired, placing them on a nutrient medium, and allowing the seeds to germinate and grow into plants. Leaves, stems, roots or other organs may then be excised and placed on a medium which will induce callus formation (Fig. 1). When the callus is large enough, it is subcultured and tissue cultures from leaves, stems and roots may be obtained. This procedure is the easiest, most efficient, and commonest method for establishing cultures of plant tissue.

Culture Media

The culture medium is an important aspect of establishing a plant tissue culture. The basal medium used consists of the major cations and anions (K, Mg, Ca, phosphate, sulphate, and nitrate), trace elements, sucrose and the vitamins B_1, B_6, and niacinamide. If a callus tissue is desired, a plant hormone is added to this medium. (We generally use 2,4-dichlorophenoxyacetic acid or *p*-chlorophenoxyacetic acid at 6 ppm). As many plant tissues have unusual growth factor requirements, a complex source of vitamins, amino acids, organic acids, and unknown growth factors is usually added to the basal medium. We routinely use coconut milk, yeast extract, or malt extract.

Fig. 1. Formation of callus tissue in cotton cotyledons on medium containing coconut milk and 2,4–D.

Although defined media are used for many plant tissue cultures for experimental studies, we are interested also in crude sources of nutrients for the larger scale work we have projected. We prefer to use easy-to-obtain, cheap sources of constituents for the media. Coconut milk, so useful at the laboratory level, is not always readily available and, under present circumstances, not cheap. Work with crude raw materials such as soybean meal, cornsteep solids, casein hydolysate, whey, yeast extract, malt extract, and others showed that certain tissues can be grown successfully using these materials. For example, rose, which is one of our best tissues for large-scale growth, grows extremely well on a basal medium containing 2,4–D and a mixture of yeast and malt extracts (0·1 per cent each).

Types of plant tissue cultures

Almost all of the parts of a plant have been induced to form tissues *in vitro*. In our cultures we have those from leaves, stems, roots, tubers, pollens, prop roots, cotyledons, petioles, and endosperm.

As for representation of the plant kingdom, mosses, ferns, gymnosperms, monocots, and dicots are all included. Genetically, tissues having 1, 2, and 3 sets of chromosomes are represented.

Growth under Submerged Conditions

From a fundamental as well as from a practical point of view, it is desirable to grow plant tissue cultures as cell suspensions under what is now generally referred to as 'fermentation' conditions. Our results with pole bean[8] showed that plant material can be grown as cell suspensions indefinitely (Figs. 2 and 3).

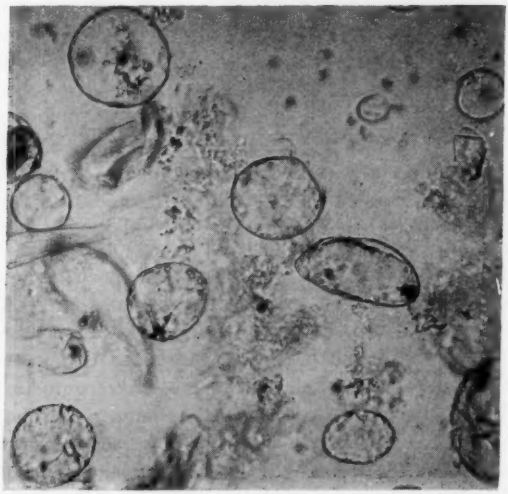

Fig. 2. Cell culture of pole bean hypocotyl 6 weeks after inoculation. × 177

Since that time, these techniques, and improvements on them, have been extended to many other plants both in our laboratory and elsewhere.[1, 5, 6, 8, 12]

Routine culture work on solid medium is carried out in 1 × 6 in. test tubes with 20 ml of medium. Most of the preliminary submerged growth studies are done in 300-ml Erlenmeyer flasks with 100 ml of medium on a rotary shaker.

Our first work with larger volumes was carried out in Fernbach flasks containing one litre of medium. The growth rates obtained were encouraging, and we started further scale-up studies. The principal tissue used for larger volume culture was obtained from Paul's Scarlet rose stem. This tissue was selected because of its rapid growth on solid media, its excellent growth in liquid culture, and because it will grow on media with coconut milk replaced by malt and yeast extracts.

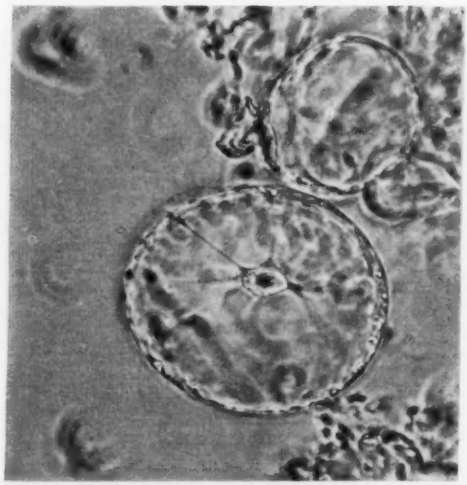

Fig. 3. Cell of pole bean hypocotyl, enlarged, same culture as in Fig. 2. Phase contrast ×488

A 20-l. carboy is used in the next stage of the scale-up. The inoculum for the carboy is prepared by taking the tissue culture from a test tube and placing it in liquid medium in a 300-ml Erlenmeyer flask. This culture is then agitated on a New Brunswick gyrorotatory shaker. After one week's growth, the contents of one flask (the culture in 100 ml of medium) is transferred to a Fernbach flask containing 900 ml of medium. This flask is also aerated and agitated by rotatory shaking. After an additional week's growth, the Fernbach culture (1,000 ml) is inoculated into

a 20-l. carboy containing 9 l. of medium. This is the final stage of the scale-up for the carboy. For purposes of aeration and agitation of the culture during growth, the carboy is fitted with stainless-steel tubing to receive compressed air, which is sterilized by passage through two Seitz filters. (Sterilization of air by packed glass wool in cylinders is also possible.) In addition, appropriate connections are included for an air outlet, medium supplements and sampling. In all of these manipulations, aseptic procedures must be followed (Fig. 4).

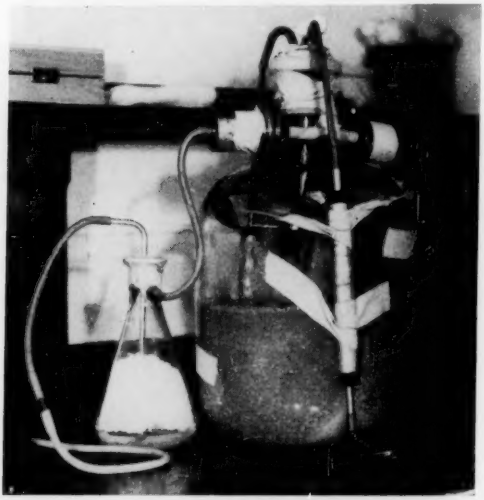

Fig. 4. The carboy culture apparatus. Aeration and agitation are accomplished by the introduction of compressed air through a side-arm trap and then through 2 Seitz filters, which sterilize the air

Samples of the carboy culture were made at weekly intervals and compared with results obtained from the same tissue grown on a New Brunswick gyrorotatory shaker in 300-ml Erlenmeyer flasks containing 100 ml of culture. These results indicate an accelerated rate of growth in the carboy for the first two weeks. Growth values (final wet weight/initial wet weight) of 5·5 and 14·9 are attained by the tissue grown in the carboy system after one week

and two weeks, as compared to growth values of 1·1 and 1·9 for the same period with the shaker cultures (Fig. 5).

The difference in growth rates between these two systems during the first week is due to the method of aeration. At the end of the first week of growth, an addition of 2 l. of medium was made to the carboy; the effect of this supplementation is a sustained rate of growth during the second week. When no further additions are made, the rate of growth drops off sharply, as shown by the growth values for the third and fourth weeks.

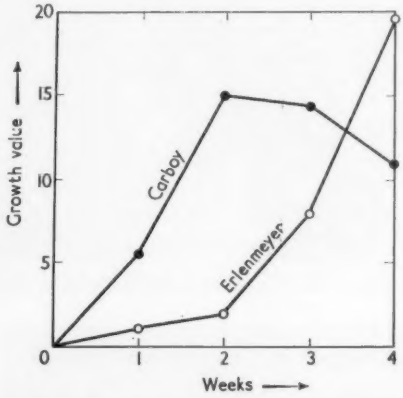

Fig. 5. Growth curves for Paul's Scarlet rose stem tissue grown in carboy culture and in Erlenmeyer flasks

Other tissues also grow well in this carboy system of culture. The data available so far for tissues from *Ilex* stem[9] (Fig. 6), *Lolium* endosperm,[11] and *Ginkgo* pollen tissue,[13] for example, yielded 2 lb of cells after four weeks in carboy culture.

Following the carboy results, we have started pilot plant runs utilizing the information gained. From the 10 l. of medium in the carboy the volume was increased to 30 l. of medium using a stainless-steel tank and finally to 135 l. of medium in a pilot plant tank. Although the number of small tank and pilot plant runs has not been many to date, the results are encouraging. The highest yield was 7 lb wet weight.

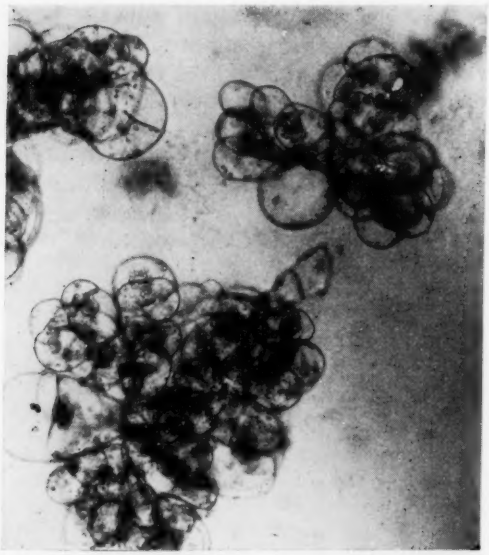

Fig. 6. Cell clumps of *Ilex* (holly) stem tissue grown in carboy culture

Analyses for Essential Compounds

With this progress in overcoming the problem of growth, we turned our attention to the tissues and cells and what they contained. In collaboration with Dr. L. H. Weinstein, of the Boyce Thompson Institute, analyses of the tissues as grown *in vitro* were compared with those of the same part from which they were derived, of the plant grown in nature. The comparisons were made for free amino acids, combined amino acids, non-volatile organic acids, sugar, and nucleic acids. Since a tissue culture represents a segment of the whole plant, diverted in its normal function and removed from some of the controls of the parent plant, both similarities and differences were anticipated and subsequently were found. The most complete work to date has been with *Agave toumeyana* leaves and a culture from these leaves. However, these results are now published and will not be presented in detail.[14] Based on these biochemical analyses, it can

be concluded that the tissue culture is cytologically, physiologically, and biochemically distinct from the parent plant part. Outstanding differences were found in the content of free and combined hydroxyproline, the ratios of malate to citrate, of ribonucleic to deoxyribonucleic acid, and of free glucose, fructose, and sucrose. A number of unknown organic and amino acids were found. Some were common to both the tissue culture and the plant parts and others were found in only one.

Secondary Compounds

Secondary plant compounds, which include alkaloids, steroidal sapogenins, terpenes, and others, are the most interesting and important group from a practical point of view. We have just started to study this group of materials.

Alkaloids are known to be produced *in vitro* by callus cultures of *Atropa belladonna*.[15] We know of no work reporting biosynthesis of steroidal sapogenins by tissue cultures.

We have two cultures from plants which produce sapogenins in nature: *Agave toumeyana* leaf (hecogenin) and *Dioscorea composita* tuber (diosgenin)[9] (Fig. 7). We are now using these tissues as

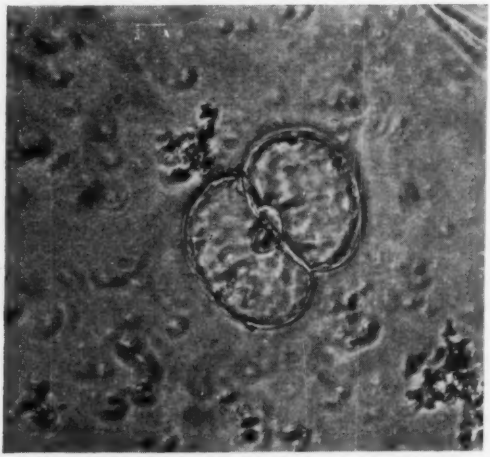

Fig. 7. *Dioscorea* yam cell in culture after division. Phase contrast × 488

research tools in the study of the biosynthesis of steroidal molecules. This is being approached through the use of steroid precursors and through the study of the physiology of these tissues. It is more than passingly interesting to us that in *A. toumeyana* the sapogenin is found in the green part of the leaves and the flowering and fruiting structures which are exposed to the sun, and nowhere else, whereas in *D. composita* the sapogenin is found only in the tuber which is never exposed to the sun; none is in the exposed parts of the plants. Furthermore, hecogenin is present in *A. toumeyana* in the maternal parts of the fruiting structure, i.e. seed pod, placenta, seed coat, but not in the embryo. Yet within a few hours after germination, hecogenin can be detected in the embryo. In *D. composita*, the sapogenin is not in the embryo before, during, or after germination; it is not in the young seedling nor in the young tuber when it is first formed. Physiologically these two steroidal molecules are probably unrelated. Because of its immediate synthesis during seed germination, hecogenin is very likely involved in growth, as opposed to diosgenin which appears late and is the result of growth. We hope that our tissue culture approach to the problem will throw some light on this question.

Perspectives

We feel that advances of the type discussed here together with the results from other laboratories have brought us to the threshold of potential, practical utilization of plant tissue culture. The results of the growth experiments reported here plus cell generation time studies reported recently [4] strongly suggest that growth will not be the bottleneck.

It is our feeling and hope that these techniques can and will be used for the study of basic cell function, for the production of known desirable compounds, for the production of new compounds, in transformation reactions, and in mixed fermentations. Probably the most exciting prospects lie in the possibilities that such cells, freed from the restraints of being part of a multicellular, multifunctional organism, will, in effect, be a new group of microorganisms with all the capabilities which this encompasses.

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Mass Algal Culture in Space Operations

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Summary. A system is described which uses photosynthesis to regenerate oxygen from exhaled carbon dioxide in sealed cabins in the absence of gravity. Design considerations as to choice of algal strain, illumination, nutrients and gas-exchange methods are discussed. Details are given of such an apparatus, of laboratory size, used to evaluate selected semi-permeable gas-exchange membranes. Data is presented and extrapolated to estimate size and weight of a possible manned space system.

Introduction

The use of photosynthesis as a means of regenerating oxygen from carbon dioxide in space cabins has been shown to be biologically feasible. The earlier reports of Myers[1] as well as more recently reported work of Gafford and Craft[2] and of Burk[3] all support the hypothesis that liquid cultures of microscopic algae can be relied upon to provide adequate quantities of photosynthetically produced oxygen with the concomittant organic fixation of carbon dioxide.

General Design Considerations

As a working component in a sealed cabin, the photosynthetic gas exchanger requires development. Some of the problems yet to be solved are mainly biological in nature. Examples of these are the problem of maintaining very high culture densities and growth rates under high levels of illumination and the problem of developing an adequate method for using human wastes as a source of nutrient for algal cultures. Other problems may better be classified as engineering. Much of the instrumentation and regulation of such a system, for example, can be defined in terms of engineering parameters.

Choice of Algal Strain

Because photosynthesis is a catenary series, there is unquestionably some reaction which limits the overall rate. The reaction in the series which is rate limiting will depend on the prevailing conditions. At low light intensities, the photochemical reaction will be limiting. As light intensity is increased, the supply of carbon dioxide may become a limiting factor. If both light intensity and carbon dioxide concentration are adequate, then one of the enzymatically regulated chemical reactions in the sequence may become limiting. In this case, temperature becomes a factor, the overall rate increasing with temperature up to a point where damage to some cellular constituent results. Most strains of algae have temperature optima for growth and photosynthesis near 25°C. As a generalization, it may be said that these ordinary strains are characterized by light saturating intensities of from 400 to 1,000 ft cd. This means that when all factors are arranged at optimum conditions, some reaction other than the photochemical reaction is rate limiting and an increase in illumination over the saturating intensity results in no increase in growth or photosynthesis.

There is a group of algal strains known as thermophiles with temperature optima for growth and photosynthesis near 40°C. Although there is insufficient information to justify a generalization, it may be said that the thermophilic strains so far studied are characterized by higher growth and photosynthetic rates and higher light saturating intensities. The best studied of these is the 'Sorokin' strain of *Chlorella* which has a growth rate some three times that of ordinary mesophilic *Chlorella* and is light saturated at an intensity of 1,600 ft cd.[4] Also of interest is Kok's *Scenedesmus* 3.[5] Both of these strains can be grown at 40°C at the rate of about five doublings per day.

Light Source

Within the limits imposed by the characteristics of any particular strain of algae, the problem of illumination requires engineering study. The basic choice is between artificial light or direct illumination with sunlight. Superficially, there seems to be little

question. Properly managed algal cultures are characterized by practical efficiencies of the order of 20 per cent or slightly less. If thermal or visible energy is first converted to electrical energy, then reconverted to visible light, from 96 to 97 per cent of the energy is lost. Actually, the choice can be made only in terms of some specific application.

If we consider, for example, a one-man photosynthetic gas exchanger in an orbiting satellite, we find that we can produce sufficient oxygen if the input of visible light energy is of the order of 14 to 15 thousand kilogram calories per day. In space, in the vicinity of the earth, photosynthetically effective energy is delivered at this rate over an area of about 1·2 m^2. Unfortunately, this energy flux is some ten times the saturating intensity for even the thermophilic algae. In other words, even with intermittent integration of light by means of turbulence, a one-man gas exchanger will have to have an exposed area of something greater than 1 m^2 and not more than 10 m^2. This is not an unreasonably large area, but it is based on the assumption that the gas exchanger will be illuminated continuously. Only very special orbit profiles or lunar locations will allow this. As more efficient solar converters and light-weight nuclear or isotopic power supplies become available, there will be applications where artificial illumination will be indicated. Furthermore, in laboratory experiments the use of artificial light is almost essential because of the unreliability of natural illumination at the surface of the earth. Most of the experimental work in our laboratories is done with fluorescent lamps because of their higher efficiency and relatively cool nature. High intensity tungsten filament lamps and pulsed gas discharge lamps are also potentially interesting.

Nutrients

The source of nutrient for the cultivation of algae in photosynthetic gas exchangers will undoubtedly be processed human wastes. It is entirely reasonable to assume that an adequate medium for growth of the algae can be developed from human waste products. Preliminary laboratory and field tests have confirmed this expectation.

The characterization of the optimum method for processing

human wastes prior to their incorporation into the algal system represents one of the major research projects which must be undertaken in support of the closed ecology. Present knowledge does not dictate whether a biological waste processing system will be best or whether purely physical and chemical methods can be employed. *Chlorella* can be grown to reasonable densities on the effluent from a small anaerobic digestor and it may prove advisable to process the urine in such a way that urea is not destroyed or oxidized. This substance represents the major amount of excreted nitrogen. If it can be held in the reduced state, the nitrogen metabolism of the algae can be more efficient and the ratio of oxygen evolved to carbon dioxide taken up will more closely balance the human respiratory quotient.

Concurrent with waste processing studies, it is necessary to study the nutritional characteristics of harvested algae. The chemical energy fixed in the photosynthetic mechanism of the algae must be preserved and must ultimately appear as useable food energy for the human component.

With urea as the nitrogen source, about 600 g of algae will be produced per day in the production of the oxygen necessary for one man. The nutritional composition of the green algae is usually given as 50–60 per cent protein, 20–30 per cent carbohydrate, and 10–20 per cent fat. A critical examination of the experimental conditions under which these figures are obtained indicates that these are related to a specific set of growth conditions. In general, when *Chlorella* or *Scenedesmus* is grown under conditions of low light intensity or intermittent illumination and high or at least non-limiting nitrogen concentrations, the above values pertain. If illumination is continuous, or the intensity of illumination is increased, or the concentration of available nitrogen decreased, there is a shift in the nutritional content of the algae, the protein concentration decreasing while carbohydrate and fat concentrations are increased. By the proper choice of illumination intensity and concentration of available nitrogen, it is possible to obtain algae having a composition of approximately 15–20 per cent protein, 50–60 per cent carbohydrate, and 25–35 per cent fat.[6] The available information indicates that the minimum daily requirement for all known human vitamins will be contained in the 600 g of algae.

Chamber Design

In designing culture chambers, the following factors have been considered:

(a) Maximum absorption of light-energy by the algal culture is accomplished by direct immersion of the light source. Losses due to reflection and absorption by container walls are thus eliminated.

(b) Since continuous illumination of cells is not required, intermittent illumination through controlled turbulence of the culture enables film depths substantially greater than those calculated to be used.

Tube-type lamps, immersed axially in cylindrical culture containers, are best adapted to realize the above conditions.

As 'cold light' is not yet available, the problem of temperature control of the culture is a major one. Lamp sources of high efficiency in terms of lumens *vs.* caloric output must be used, within the limitations of spectral requirements.

Selection of algae strains having their optimum photosynthetic activity significantly above ambient temperatures permits the design of culture units to take advantage of heat loss to the surrounding environment. Thus, a suitable thin-walled culture unit may establish an operating equilibrium temperature somewhat below the cultural optimum, requiring only partial insulating to achieve optimum temperatures.

Another problem that must be solved in designing photosynthetic gas exchangers for use in satellites is that of zero gravity operation. This problem results from the necessity of growing algal cultures in contact with air for prolonged periods of time in the absence of an effective gravitational field.

Engineering analysis and experimentation indicate that a device employing a semi-permeable membrane to create a controlled gas–liquid interface between air and algal suspension can be used. Its operation may be thought of as that of a kind of 'reverse lung'. I will describe our current efforts in this area in some detail.

Experimental

Apparatus

Fig. 1 is a drawing of such a device. A $\frac{3}{8}$-in. thick sheet of plexiglas 15 in. × 18 in. is milled with a $\frac{1}{4}$-in. continuous groove

back and forth in the long direction. The grooves are separated by $\frac{1}{16}$-in. lands except for two wider lands used for bolt holes. The channel is approximately 60 ft long. It is connected to the outside of the panel by holes through the edge at each end of the channel. A second panel is grooved as a mirror image of the first. A sheet of the film to be tested is placed between the two panels which are greased around the edges and on the bolt lands with stopcock grease. The assembly is held together by a series of bolts around the edge and in the bolt lands. In effect, the assembly represents two $\frac{1}{4}$-in. square channels 60 ft long separated by the membrane material. The volume of each channel is approximately 700 ml.

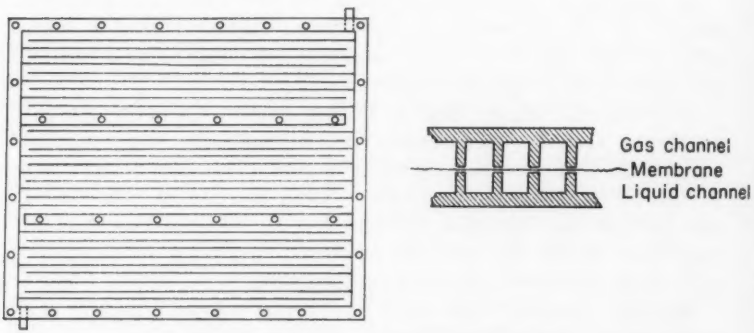

Fig. 1. Drawing of the gas exchange panel and cross section of complete assembly

This device was designed to test the principle of gas exchange through membranes and to serve as a test apparatus for a variety of films under the limiting conditions of temperature and pressure imposed by the biological material in the liquid phase. The collection of data from this device is aided by a number of instruments which feed information to a multi-channel strip chart recorder. Fig. 2 is a diagram of the overall system as it stood when these experiments were done.

The algal suspension is pumped through the channel by means of a small pump. About 50 ml of the suspension is contained in a tube which contains a thermistor and an extra outlet for sampling any gas that accumulates in the liquid phase of the system.

Air is pumped through a 500-ml ballast bottle into the panel by means of another pump. From the panel, the air passes through a liquid trap having a capacity of one litre and through a water-cooled condenser which is maintained at 10°C. The purpose of the trap is to catch the algal suspension if the membrane ruptures. The partially dried air is passed to the pump through a CO_2 analyzer which consists of a Veco temperature-compensated thermal conductivity cell in a bridge circuit. A separate loop feeds air continuously from the ballast bottle through a flowmeter and oxygen analyzer. Potentiometric type pressure transducers are connected to both the high and low pressure sides of the air system.

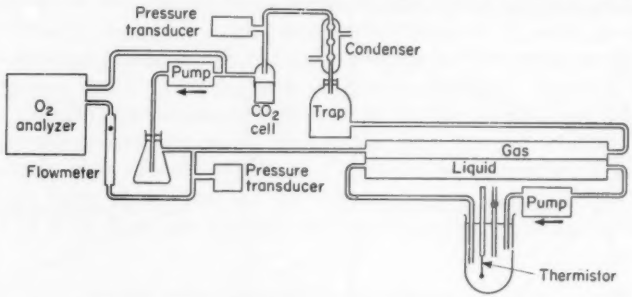

Fig. 2. Block diagram of the experimental system

Culture System

The high temperature Sorokin strain of *Chlorella pyrenoidosa* was grown in urea medium in 2·5-1. chambers internally illuminated with daylight fluorescent lamps. Cells were harvested by centrifugation, washed in urea-free medium and resuspended in fresh medium containing from 0·5–1 g of urea per litre. The final concentration of algae was approximately 1 per cent (v/v).

This suspension was introduced into the liquid channel of the gas exchanger and all gas bubbles were eliminated from the liquid phase, to provide hydraulic continuity. The panel was illuminated with a bank of ten 15-W fluorescent lamps. In these experiments, the temperature of the algal suspension was controlled by a fan moving air across the lamps and around the panel. At the beginning of each experiment, the gas phase consisted of air enriched to approximately 14 per cent with CO_2.

Results

The two most promising membrane materials tested to date are polyethylene and oriented polystyrene. The results of an experiment in which 0·5 mil polyethylene was used as a membrane and one with 1·5 mil polystyrene are shown in Fig. 3. These results represent an oxygen transfer rate of approximately 43 cc. per hour for the polyethylene and approximately 11 cc. per hour for the polystyrene. The very thin polyethylene works comparatively well insofar as gas exchange capability is concerned; however, it is not satisfactory from the mechanical standpoint. The film ruptured after only a few hours service, allowing the algal suspension to be drawn into the air channel.

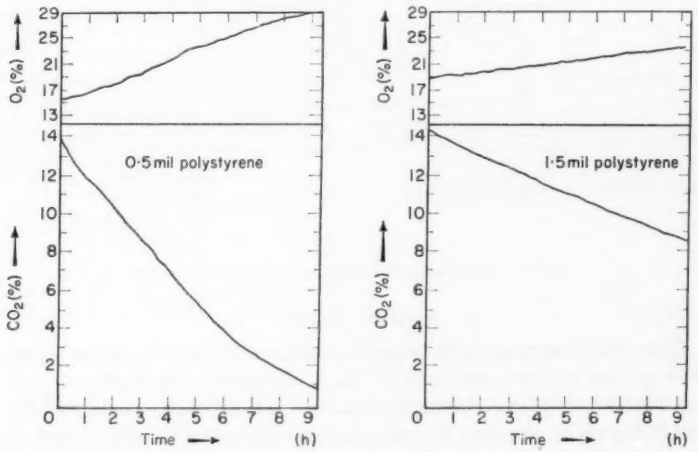

Fig. 3. Comparison of change in O_2 and CO_2 percentage in air phase with 0·5 mil polystyrene

Oriented polystyrene of 1·5 mil was substituted because of the superior mechanical characteristics of this material. The rate of gas transmission is lower than that of polyethylene. With this film in the panel, a significant amount of gas collects in the liquid phase. This gas is trapped in the small tube in which the thermometer is mounted. Samples withdrawn at this point contain no carbon dioxide and from 50 to 70 per cent oxygen.

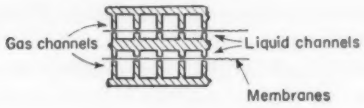

Fig. 4. Cross section of panel modified to provide 2× area of film

Fig. 4 is a drawing of the cross section of a modification of the panel. The panel was rebuilt to accommodate two sheets of film by milling $\frac{1}{8}$-in. deep grooves in both sides of a third sheet of plexiglass. These grooves terminate in common outlets. The grooves in the centre panel serve as the liquid channel and the grooves in the two outside panels are connected in series to serve as the gas channel. The liquid volume is the same as in the previous model, but the area of film is doubled. Results from an experiment conducted with this device employing 1·5 mil polystyrene are compared with those from the experiment with a single area of polystyrene in Fig. 5. The rate of exchange is about twice that observed for the single area panel being 11 cc. per hour in that case and 23·5 cc. per hour with double the area.

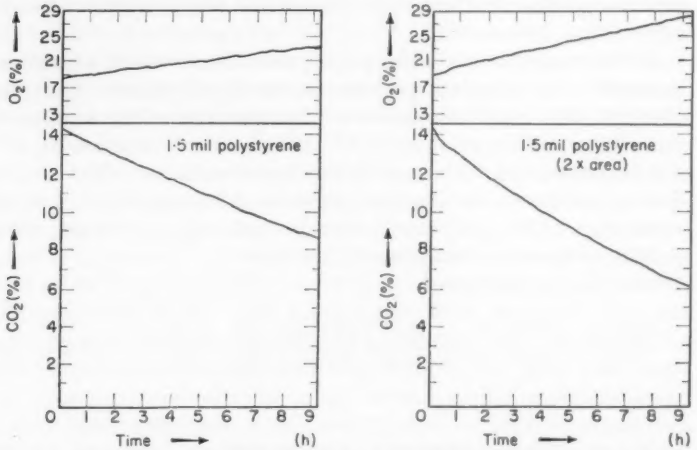

Fig. 5. Comparison of change in O_2 and CO_2 percentage in air phase with single and double area of 1·5 mil polystyrene

Some general conclusions can be reached from these experiments with all films successfully tested. First, with the volume of algal suspension and level of illumination employed, the CO_2 diffusion rate across the membrane is the limiting factor in the overall photosynthetic rate. Secondly, oxygen diffusion proceeds at a lower rate than does oxygen evolution by the algae. Finally, with non-wettable films such as polyethylene and polystyrene, transfer of water vapour is so low as to be almost negligible. Theoretical prediction of the potential rate of exchange of oxygen can be made from the data provided by the film manufacturers. For polyethylene, oxygen permeability is given as 430 cc./24 h × 100 in.^{2} × mil thickness × atmosphere; for oriented polystyrene, the value is 210 in the same units.

The pressure differential was not measured accurately, but can be estimated roughly. In experiments with both films the absolute pressure in the gas phase averaged approximately 620 mm Hg. With an average of 23 per cent O_2 this represents an estimated average pO_2 of 143 mm Hg. In the experiments with the polyethylene, the pressure in the liquid phase remained close to ambient, that is approximately 620 mm Hg. It is assumed that the gas on the liquid side of the film was essentially 100 per cent O_2. Thus, in these experiments the pO_2 is estimated to be $620 - 143 = 447$ mm Hg.

In the experiments with polystyrene there was a significant increase in the absolute pressure in the liquid phase. Only one attempt was made to measure this pressure with a mercury manometer (differential to ambient) and this determination cannot be considered to be necessarily representative. The reading was equivalent to an absolute pressure of 846 mm Hg. A rough estimation of the pO_2 would then be $846 - 143 = 703$ mm Hg.

The predicted exchange rates are then
for 0·5 mil polyethylene:

$$\frac{430 \text{ cc.}}{\text{day}} \times \frac{180 \text{ in.}^2}{100 \text{ in.}^2} \times \frac{1 \text{ mil}}{0{\cdot}5 \text{ mil}} \times \frac{477 \text{ mm Hg.}}{760 \text{ mm Hg.}} \times \frac{1 \text{ day}}{24 \text{ h}} = 40{\cdot}5 \text{ cc./h}$$

for 1·5 mil polystyrene:

$$\frac{210 \text{ cc.}}{\text{day}} \times \frac{180 \text{ in.}^2}{100 \text{ in.}^2} \times \frac{1 \text{ mil}}{1{\cdot}5 \text{ mil}} \times \frac{703}{760} = 9{\cdot}75 \text{ cc./h}$$

In both cases, the predicted rate is fairly close to, but lower than the observed rate. The discrepency is probably due to inaccurate measurement of the pO_2 in the system. The results obtained so far have established the validity of the principle that a gravity independent gas–liquid interface can be established across a semi-permeable medium in such a manner as to allow for appreciable exchange of CO_2 and oxygen while limiting the transfer of water vapour.

Some other materials, as yet untested, which have possible application in this type of device include irradiated polyethylene, porous teflon, silicone rubber, woven stainless-steel cloth, and Corning porous Vycor glass. Acetate film and cellophane have been tested and both are characterized by excessively high rates of water transmission and generally poor mechanical characteristics. Mylar, even as thin as 0·25 mil, while mechanically superior, is characterized by very low permeability to both CO_2 and O_2.

The porous glass may be obtained as tubes 9 mm in diameter with 1 mm thick walls. The total length of tube obtained is approximately 5 meters providing a total area of approximately 1,400 cm^2 or about 217 in^2. This compares favourably with the surface of 1,160 cm^2 characteristic of the single panel described above. On the basis of the data provided by Corning, the predicted rate of exchange for O_2 through a thickness of 1 mm having an area of 217 in^2 in between 3,000 and 5,000 cc./h, some 100 times that predicted for the same area of the polyester films. However, one major difference in the physical characteristics of the film and the glass alters this prediction drastically.

The polyester films are non-wetting and their action in this application appears to be essentially a diffusion phenomenon. By contrast, preliminary experiments indicate that the porous glass is extremely hygroscopic. It will absorb as much as 25 per cent of its weight of water (as compared to less than 1 per cent for the polyester films). For this reason, it is expected that the exchange of oxygen through this material will be largely a function of rate of solution and dissolution of O_2. One possibility for improving this situation might be to coat the glass with some hydrophobic substance which would serve to prevent saturation of the glass with water without altering the gas permeability. Other materials are 0·5 mil Teflon and 5 mil silicone rubber.

Predicted diffusion rates for oxygen in the experimental model are 160 cc. per hour for the Teflon and 1,600 cc. per hour for the silicone rubber. The later figure is extremely promising. Provided the observed rate equals the predicted rate, the exchange rate per unit area will be higher than the maximum rate of O_2 produced per unit area at solar levels of illumination and membrane permeability will not be the limiting factor in the design of the gravity independent photosynthetic gas exchanger.

Design Estimate

From the results so far obtained in our own and other laboratories, it is possible to estimate approximately the dimensions of a representative one-man photosynthetic gas exchanger. If we consider only the special case where a reasonably sized area on a satellite or lunar base can be continuously illuminated, then the area required to effectively absorb the required energy is estimated at 5 m^2. This assumes sufficient turbulence in the outline to integrate about 50 per cent of the available illumination in excess of the saturating intensity. It is predicted that the silicone rubber film will exchange oxygen at about twice the rate at which it is evolved. In other words, 5 m^2 of 5 mil silicone can be expected to transfer about 1,600 l. of O_2 per day.

Following closely the design of the laboratory prototype, a culture volume of about 35 l. is required. If this can be maintained at densities of between 2 and 8 per cent wet volume per volume with a growth rate of only two doublings per day, then the necessary oxygen can be obtained. The mean depth of the liquid phase would be 0·7 cm.

Engineering requirements dictate that no more than about 80 per cent of the total area in a panel of this type should be active. The balance of the area is required to maintain structural integrity.

The panel might have a circular configuration. If so a diameter of 2·82 m (9·25 ft) is needed. The thickness of the panel need be no more than 1 in. and its weight will be between 150 and 250 lb. The accessory equipment necessary to process waste and to harvest the algae is estimated to weigh another 100 lb. Thus,

the minimum estimate for weight of the system is 250 lb. Fig. 6 is a comparison of the weight of such a system with the weight of a conventional atmospheric conditioning system. Therefore a properly designed photosynthetic gas exchange system might have a competitive advantage when operational periods exceed about 33 days.

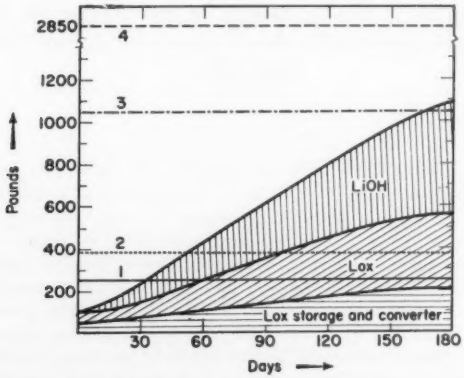

Fig. 6. Estimated weights of photosynthetic gas exchange systems and liquid oxygen–lithium hydroxide system as a function of time in orbit. Horizontal lines as described in text

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Studies on Large-Scale Methods for Propagation of Animal Cells*

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Summary. A new strain of cells, designated as PD–MEK–I, and derived from the kidneys of a rhesus monkey embryo, has been isolated and utilized as a prototype for mass propagation in suspended cell cultures, using techniques similar to those developed for antiobiotic fermentations. The use of the large-volume units for cultivation of tissue cells has been applied. These units are useful for preparing masses of cells for studies of cellular antigens, virus assays, antitumour cytotoxicity studies, and other biologically active materials, as well as seed for continuing cultures. In addition, other methods of roller bottle cultures, shaker flask cultures, spinner cultures, and disposable 1-litre prescription bottles, have been modified so that over 35 different human and animal cell lines are being maintained in continuous culture. Any one of these procedures may be modified for preparing and handling large numbers of replicate cultures as well as for maintaining stock seed cultures. The characteristics of growth of cells utilizing these methods are similar to those encountered in microbial populations and the principles of antibiotic fermentation have been applied appropriately.

Significant advances have been made in the mass propagation of cells over the past few years. There can be little doubt that techniques for large-scale continuous cultivation of mammalian cells will continue to be developed, with the result that new and unexplored fields will be opened for the application of tissue culture. In fact, many of the current procedures clearly show that mass propagation of tissue cells is now of practical value.

* Presented to the Fermentation Subdivision Meeting of the American Chemical Society, in Atlantic City, New Jersey, September 13–18, 1959.

Much progress has been made from the early method of cell culture which consisted of embedding small primary explants in a plasma clot on a glass surface, and gently rotating the culture in nutrient medium. At best only small amounts of tissue cells were ever available with this slow and time-consuming process. Subsequently, Carrel[1] developed the technique for cultivation of cells as a monolayer on glass surfaces which afforded much larger quantities, but still necessitated critical manipulations. The simplification and refinement of these basic tissue culture procedures have provided a number of methods which are applicable to the large-scale cultivation of animal tissue cells.

It is the purpose of this report to summarize various techniques for preparing and handling replicate cultures in a number of different types of container. The potential value and application of these methods for biological investigations is the result of a systematic survey undertaken early in the development of polio vaccine to select an efficient and economical technique for the cultivation of primary monkey kidney cells that could be available for production of polio-virus. Likewise, these or similar methods are being applied to the production of a number of viral vaccines, hormones, other biologically active materials, and to preparing large volumes of cells for antigenic analysis and fractionation.

Initially, classical roller tubes, modified Maitland type cultures, and a number of other procedures were investigated, but the method of choice was cultivation of trypsin-dispersed cells as a monolayer on a clean glass surface in stationary culture. For this purpose it was necessary to standardize the containers, and a one-litre (32-oz) prescription bottle used for dispensing liquid pharmaceuticals was selected as the standard unit. Smaller sizes consisting of 2, 4, 8 and 16 ounces are also available, and these have been utilized for maintaining stock cultures, isolation and establishing new cell lines, as well as other applications where larger volume is not required. These containers are made to rigid standards as to leaching of alkali and composition of glass, and are roughly calibrated in both fluid ounces and millilitres for convenience in inoculating, feeding, and handling medium changes. They are commercially available and it is economically feasible to utilize them as disposable items—to be used once and then destroyed. Furthermore, it was found that these bottles as received from the

manufacturer could be used for tissue culture directly, without the washing required with re-used glassware customarily employed for cultivation of cells. Before use the new bottles are rinsed once with distilled water, closed with a cotton-gauze stopper, and sterilized in the autoclave. Cultures grow in bottles prepared in this manner as well as on glassware prepared by the rigid tissue culture wash-and-rinse procedures.

While there are many advantages with the standard 32-oz unit prescription bottles, which are still utilized for production and

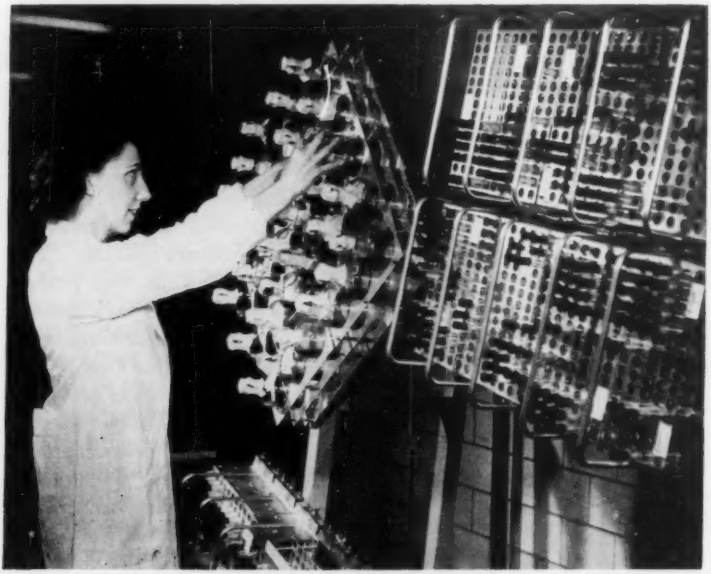

Fig. 1. Rack boards adaptable for demountable racks carrying either roller bottles or tubes. Four rack boards are rotated by a single drive unit

testing of polio-virus vaccine, alternate methods make use of roller bottles held in rotating racks. In Fig. 1 are shown the rack boards which rotate at 8–10 rev/hour carrying demountable racks of either 500 ml or 1-litre roller bottles. The cells settle out and grow on the glass. Once such a bottle is established, the culture may be

used for virus production, yield of cells, and preparation of cell mass. In addition the same rotating equipment will hold racks of roller tubes, and thus it is adaptable to a wide range of flexibility and utility.

The procedures utilizing monolayer cultures on glass have been applied to a wide variety of studies and can be recommended for routine use. However, it was recognized by many workers that mammalian cells growing *in vitro* display growth patterns quite analogous to that of microbial systems, and likewise could be propagated in suspended type cultures for special applications. As an example, the MBIII strain of mouse lymphoblasts were first to be cultivated in agitated fluid suspension in 1953 by Owens and Gey.[2] These studies were extended by Earle *et al.*,[3] who showed that a strain L mouse fibroblast could be grown in Erlenmeyer flasks on a rotary shaker. Using a similar system, Kuchler and Merchant[4] have demonstrated the value in quantitative biological studies. In extending these investigations a number of mammalian cells (HeLa, H.Ep. #2, human amnion, monkey kidney) have been utilized and successfully propagated as a suspension culture in 1- and 2-l. Florence flasks on a rotary shaker. The cultures have been maintained for up to four months with partial harvests at weekly or bi-weekly intervals when the rotary motion imparted by the device was operated at a swirl rate of 100 rev/min. One primary monkey kidney culture was maintained as a suspended cell in this fashion for eight months. The cells grown in this manner differ quite markedly from the established line on glass, being smaller, ovoid, and more compact in appearance.

More recent studies have utilized spinner cultures which were developed from an apparatus first designed by Cherry and Hull[5] in which a magnetic stirrer bar was suspended from a wire attached to a fishing swivel and permitted complete freedom of the bar in suspension for the agitation of cells. McLimans[6] has developed the spinner culture system for other cells in which gentle agitation is maintained by a suspended teflon-coated bar magnet spinning approximately at mid-fluid level. Mammalian cells have been adapted to these units from monolayer cultures more readily than to the rotary flasks. Such cultures have been useful for maintaining small volumes of cell stocks in continuous cultures with a minimum of effort. In addition, these cultures have provided

preliminary information for the growth of cells in fermentor tanks, such as are used in the antibiotic industry.

Application of fermentors to growth of mammalian cells was first reported by McLimans *et al.*[7] in 1957, in which conditions were defined for growth of L-strain mouse fibroblasts. Results of their study have established a practical and efficient system for growing mammalian cells in mass culture. This work was extended to other cell lines (HeLa, ERK) in 20-l. fermentors.[8] Utilizing these methods, particular emphasis has been placed on a line of kidney cells established in our laboratory over 16 months ago from a rhesus monkey embryo. This new cell type derived from the monkey embryo kidney has been designated as PD-MEK-I, and this strain is of particular interest because of its broad virus spectrum. It is useful, therefore, for a number of virus assays, and because of its rapid growth rate lends itself well to antitumour cytotoxicity studies, and conditions have been developed for preparing large quantities of these cells for studies of cellular antigens. This system has served as a useful model in developing conditions for growth of other cells in submerged culture. The cells were first isolated as a monolayer on glass, and maintained in this fashion for 16 months, then transferred and adapted for propagation in fermentor cultures.

A typical growth curve of a sustained culture of PD-MEK-I cells maintained in a 5-l. New Brunswick Fermentor* for 30 days is shown in Fig. 2. The culture was initiated with 2·0 l. of cells at a concentration of 200,000 cells/ml in a medium consisting of 10 per cent calf serum + 15 per cent Tryptose phosphate broth in Eagle's basal medium with Earle's buffer. The culture is agitated by means of a 4-blade impeller operating at 160 rev/min. Using this system, gassing of the culture with CO_2 was unnecessary. Cells were harvested and fresh medium added 12 times during the 30-day period with changes of 30 to 64 per cent of the total volume. On day 9 an additional 800 ml of medium were added to increase the volume from 2·0 to 2·8 l. On day 21 the volume in the fermentor was again reduced to 2·0 l. This type of growth with the MEK–I cells demonstrates the potential for mass propagation of mammalian cells in suspended culture.

* Purchased from New Brunswick Scientific Company, New Brunswick, New Jersey.

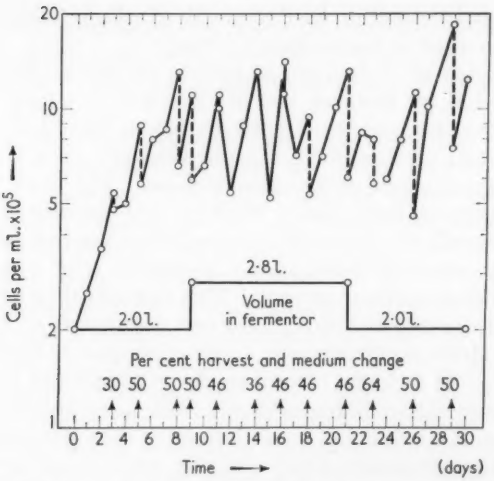

Fig. 2. Sustained culture of PD-MEK-I cells in 5-l. New Brunswick fermentor

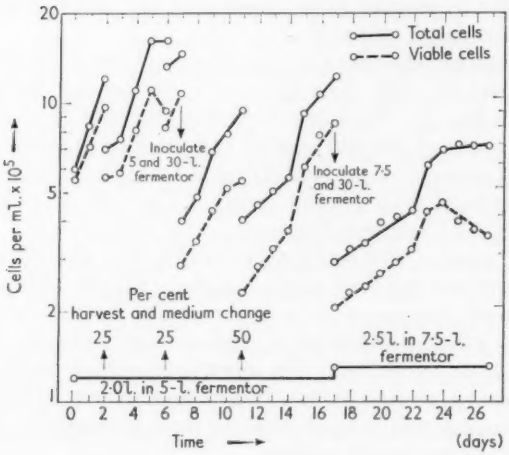

Fig. 3. Continuous mass propagation of PD-MEK-I cells in suspended fermentor cultures

Fig. 3 illustrates the possibilities for the continuous mass propagation of MEK–I cells in suspended fermentor cultures. Here a 5-l. fermentor was initiated with a 2-l. volume at a concentration of 600,000 cells per ml. There was a 25 per cent harvest and medium change on days 2 and 6. On day 7 the cell count was 1·3 million cells per ml and this was used to initiate a second 5-l. and a 30-l. fermentor. As the 5-l. fermentor culture was held further, there was a 50 per cent cell harvest and medium change on day 11 and it was possible to initiate a 7·5-l. and a second 30-l. fermentor from the yield on day 17. The growth in the new 7·5-l. fermentor is then demonstrated from the 17th to the 27th day of the experiment. The original 5-l. fermentor was used at this time for virus production. The rates of cellular multiplication were determined by crystal violet-nuclei counts in a hemocytometer. Cell viability was determined by use of vital stain counts using both neutral red and trypan blue.

Utilizing the 7·5-l. fermentor, the effect of increasing volume on the propagation of MEK–I cells in submerged culture is illustrated in Fig. 4. At a volume of 3 l. approximately 90 per cent of the total cells are viable, but as the volume is increased to 4 l., the

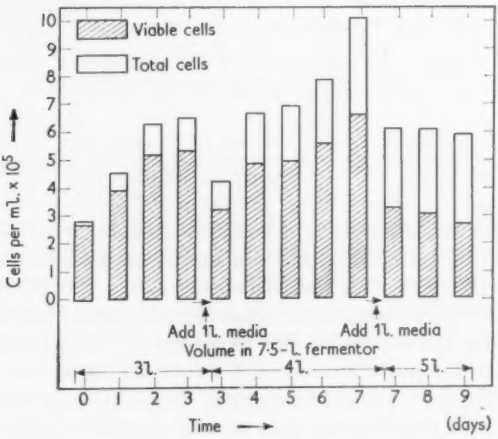

Fig. 4. Effect of increasing volume on propagation of PD-MEK-I cells in submerged cultures

number of viable cells diminishes to about 60 per cent. As the volume is further increased to 5 l. in the 7·5-l. fermentor, less than half of the cells remain viable. In this system a total volume of 3 l. has provided the optimal condition for greatest yield of viable MEK–I cells.

The utility of this type of suspended culture is demonstrated in Fig. 5 in which measles virus has been propagated in the MEK–I cells. The culture was initiated with 200,000 cells per ml and

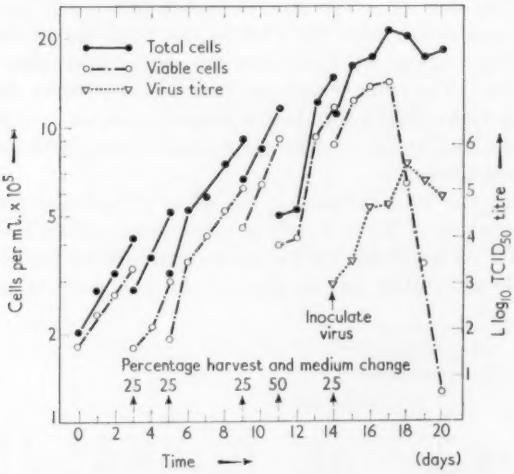

Fig. 5. Propagation of virus in suspended culture of PD-MEK-I cells (2-l. volume in 5-l. fermentor)

with a series of harvests and medium changes, it achieved a concentration of 1·5 million cells per ml on the fourteenth day. At this time the fermentor was inoculated with 1,000 $TCID_{50}$ doses of virus. It is interesting to compare the number of viable and total cells during the course of this experiment. The viable count is maintained at approximately 90 per cent during the 14 days before virus challenge. For four days after virus challenge the viable cell count is still maintained, but on the nineteenth day (five days after virus inoculation) there is a sharp drop in the number of viable cells. At this time maximal virus yields of $10^{5.5}$ tissue

culture infecting doses per ml are obtained. The viable cell count continues to decline until very few viable cells remain by six days after challenge with virus, even though the total number of cells as determined by nuclei count remains high. This probably is, at least partly, a result of rupture of the many multinucleated giant cells which are induced by measles infection.

It is apparent that total and viable cell counts must provide a useful criterion for the time to harvest such fermentor cultures in

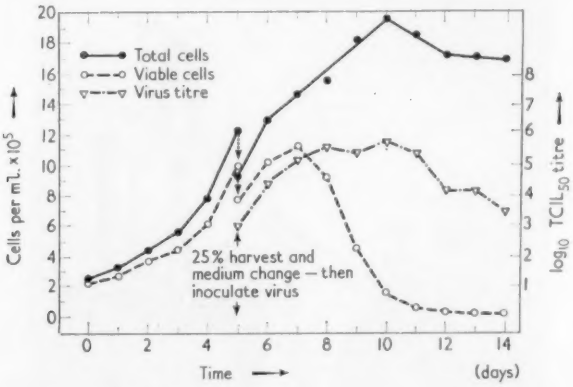

Fig. 6. Cell counts related to virus yields in suspended cultures of PD-MEK-I cells. (2-l. volume in 5-l. fermentor)

order to obtain maximal virus yields. The selection of the proper time by this index is illustrated in Fig. 6 in which a suspended culture of MEK–I cells was inoculated with 1,000 $TCID_{50}$ doses of measles virus at the fifth day, and at the time of a 25 per cent harvest and medium change. Again the percentage of viable cells was maintained for three days after challenge and the virus yield was observed to increase during this time. By the eleventh day (or five days after virus infection) very few viable cells remain and the virus titre then begins to decline. Thus, a system becomes available that could be useful for large-scale production of virus for vaccine purposes.

The use of these fermentors has been extended to large 30-l. fermentation tanks used in the production of antibiotics. Such a

crude fermentor which has been converted for mass propagation of cells is illustrated in Fig. 7. The impeller is rotated with an air-driven motor at approximately 200 rev/min. Initially aeration with 5 per cent CO_2 was utilized, but has since been found unnecessary. It is more difficult to control conditions in this type of fermentor, but the method employed is quite analogous to techniques used in antibiotic fermentations.

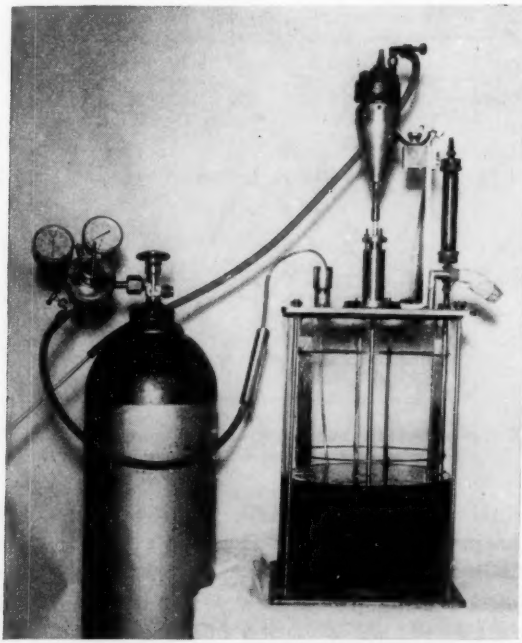

Fig. 7. 30-l. antibiotic fermentor converted for propagation of tissue cells. Impeller rotated by means of an air-driven motor

The growth curve of the MEK–I cells in this type of 30-l. fermentor is illustrated in Fig. 8. The fermentor culture was initiated with 9 l. of cells at 400,000–700,000 cells/ml from a 7·5-l. fermentor. There was a 4-l. harvest and medium change on the fifth day and a 2-l. change on the ninth day. The fermentor

was kept for 13 days when the cell mass was utilized for antigenic fractionation studies.

A continuous supply of the MEK–I cells in suspended culture has been maintained over a prolonged period and used for a wide variety of purposes. Cells have been removed from the fermentor, planted, and cultivated as monolayers on glass. They do not, in some other instances, lose their ability to adhere to the glass and,

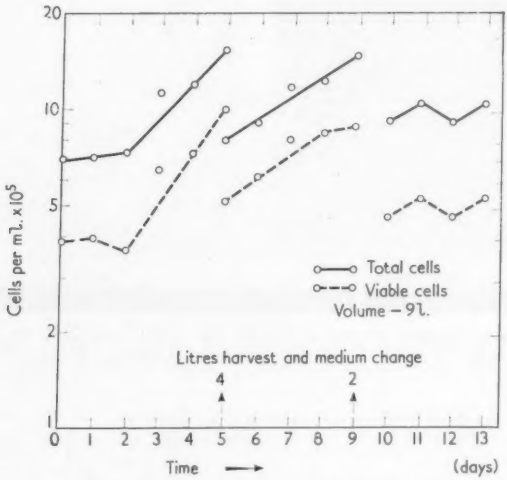

Fig. 8. Propagation of PD-MEK-I cells in 30-l. fermentor

in contrast, spread out to form a monolayer. Such a comparison of the character of the cells grown out as a monolayer culture on glass from the fermentor and from a previous monolayer culture is shown in Fig. 9. There is no clumping or granularity and a uniform monolayer is established in five or six days when bottle cultures or tubes are inoculated at 200,000 cells per ml from the fermentor. These are readily adaptable for a number of assays and other purposes in the event of monolayer cultures becoming desirable.

While the major effort has been placed on this newly established strain of MEK–I cells, the techniques have been extended to other

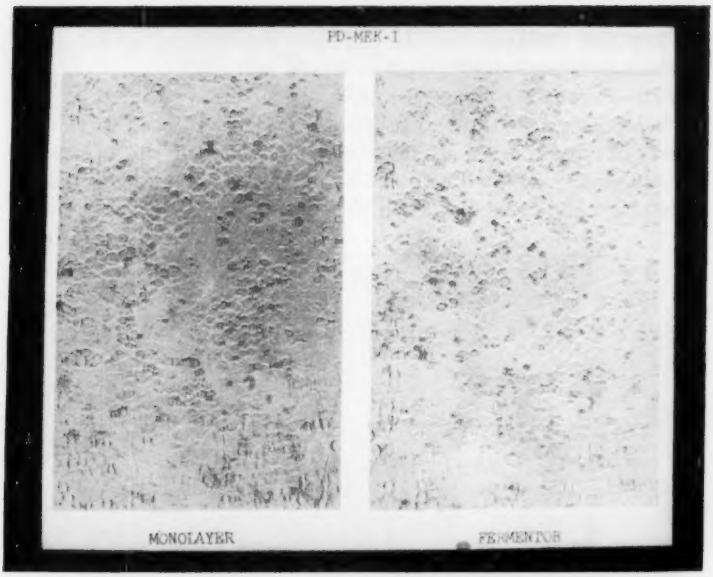

Fig. 9. Characteristics of 5-day PD-MEK-I cells cultivated on glass from established monolayer cultures and from suspended fermentor cultures

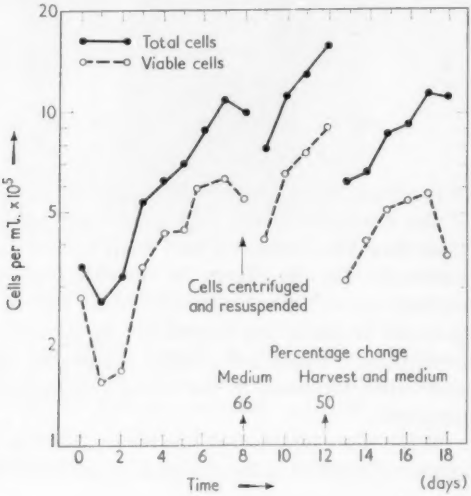

Fig. 10. Suspended culture of H.Ep. #2 cells in 7·5-l. New Brunswick fermentor (3-l. volume)

cell lines in this fashion. As an example, in Fig. 10 the method is illustrated as applied to a suspended culture of H.Ep. #2 cells propagated in a 3-l. volume in the 7·5-l. fermentor. The culture was maintained for 18 days with a 66 per cent medium change on the eighth day and a 50 per cent cell harvest and medium change on the twelfth day. The percentage of viable cells is less than with the MEK–I cell line, but the method still lends itself well to preparing masses of cells in continuous culture over an extended period of time.

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Process Report: Large-Scale Production of Foot-and-Mouth Disease Virus

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Summary. A pilot plant is described for the mass cultivation of foot-and-mouth disease virus, *in vitro*, on calf kidney cell monolayers. The pilot plant produces up to 400 litres of virus per week; it can be enlarged with ease to a sufficient capacity to meet any requirements.

Introduction

The production of vaccine at a time of widespread distribution of foot-and-mouth disease (F.M.D.) has hitherto always been insufficient owing to a lack of virus. The two sources at present available are: virus cultivated *in vivo* on the tongues of cattle destined for the slaughter-house, and virus cultivated *in vitro* according to Frenkel. Both sources are, however, limited by the availability of cattle at the slaughterer's. Though both methods have marked a fundamental stage in the production of F.M.D. vaccine, they constitute, especially in our country, an insufficient supply of antigen material.

Towards the end of 1957, favourable results were obtained by us from the culture of F.M.D. virus on kidney cells, and a vaccine was produced. Taking into account both this work and favourable experimental tests which had been reported up to that time in the literature (Bachrach *et al.*, 1955; Sellers, 1955; Mazzaracchio *et al.*, 1956–57; Petermann *et al.*, 1956; Wesslen and Dinter, 1956; Dinter and Wesslen, 1957; Guillot, 1957; Melendez *et al.*, 1957) and which have since been widened in scope and confirmed (Dinter and Wesslen, 1958; Mazzaracchio *et al.*, 1958, 1959; Armbruster *et al.*, 1959; Baldelli and Badiali, 1959; Caporale *et al.*, 1959; Castagnoli *et al.*, 1959; Rivenson *et al.*, 1959; Traub and Schwoebel 1959; Zavagli *et al.*, 1959), our Institute thought it opportune to transfer this technique from a strictly laboratory scale to an industrial one.

Indeed if this technique could be adapted to a practical level of production of the virus in large quantity, it would represent a decisive stage in the production of F.M.D. vaccine; many did not previously share the opinion that this was possible because of the numerous problems to be solved.

We now consider it opportune to communicate the ways in which the various problems have been overcome in going over from a laboratory to an industrial scale production of the F.M.D. virus using our technique.

The Laboratory Suite

The laboratory suite which at present exists in Brescia and has been functioning since 1958 is termed a 'pilot plant' because it has a weekly production of 300 to 400 litres of virus, which, though not being sufficient to attain the goal we set ourselves, already represents a reasonable quantity.

The suite is composed of the following rooms (the numbers in parentheses referring to the numbers in the accompanying plan).

A room for the de-ionizer (1) and the tank for the de-ionized water (2);

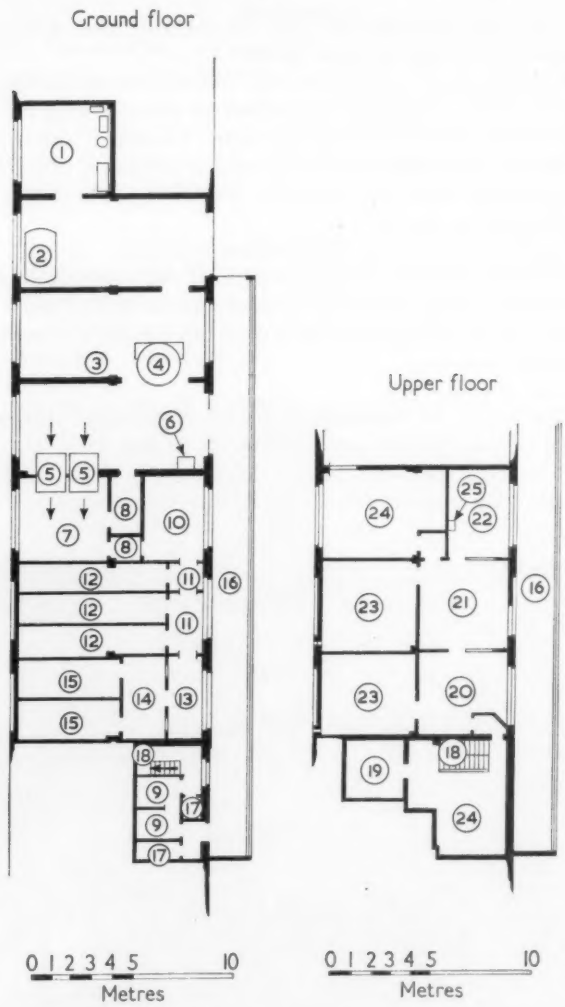
A room (3) for cleaning and sterilizing the Roux flasks. The cleaning is performed by a modified 'automatic bottle-washing machine' (4) and sterilization is in two large sterilizing ovens (5) (with a capacity of 840 Roux flasks).

A room for storage of the Roux flasks after sterilization (7) and a room for changing overalls and shoes (8).

A room (10) for inoculating the Roux flasks with the cell suspension. A communication passage (11) leads between the room for inoculation (10) and the incubators (12).

Three incubators (12) (for growth of cellular monolayers) with forced air circulation: each incubator measures 7 m by 1·35 m, and is 2·30 m high. Each incubator can hold eight trucks (described later) carrying in all 1,456 Roux flasks. A communicating passage (13) leads between the incubators and the room for the inoculation and collection of virus where microscopic observation of the monolayers in also carried out.

A room for the inoculation and collection of virus (14).



Two incubators (15) for incubation of cell monolayers after infection (dimensions in metres: $4{\cdot}70 \times 2{\cdot}20 \times 2{\cdot}30$; capacity of 12 trucks equivalent to 2,184 Roux flasks). A communicating

passage (16) runs between the room for the collection of virus and the room for cleaning the Roux flasks.

On the upper floor, which is reached through rooms for changing overalls (9) and by the stairs (18), there are the following rooms.

A laboratory (20) for the preparation of kidneys (decapsuling, chopping etc., up to the first trypsinization inclusive) (Fig. 1).

A refrigerated room (19) where the second trypsinization, according to Bodian, is carried out.

A laboratory (21) for the centrifugation of cells.

A laboratory (22) for the preparation of cell suspension, with a communicating trap door (25) between this room and room 10.

Rooms (23) of machines for forced air circulation and heating of the incubators.

Storerooms (24).

The room for the inoculation of cell suspension (10) on the ground floor and the laboratories nos. 20, 21 and 22 on the upper floor are kept air-tight and receive sterile, conditioned air from an air conditioning unit combined with a filtration system and ultraviolet lamps (6).

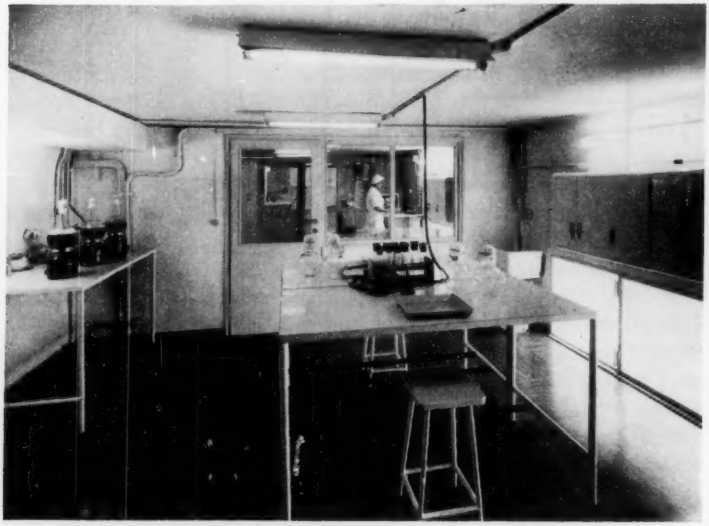


Fig. 1. Laboratory for the preparation of kidneys

Equipment

Under this heading we shall describe only the de-ionizer, the automatic washing machine for the Roux flasks and the trucks for the transport and incubation of the flasks. We consider it unnecessary to describe the normal laboratory equipment (Leitz–Waldmann microscope for chemists, magnetic stirrers, Seitz filters, automatic dispensers, etc.).

De-ionizer

The de-ionizer (Fig. 2) has an hourly production of about 3 m^3 of de-ionized water with an electrical conductivity of 1–3 micro-siemens. This de-ionizer is connected to a tank of 4 m^3 capacity which is kept full automatically.

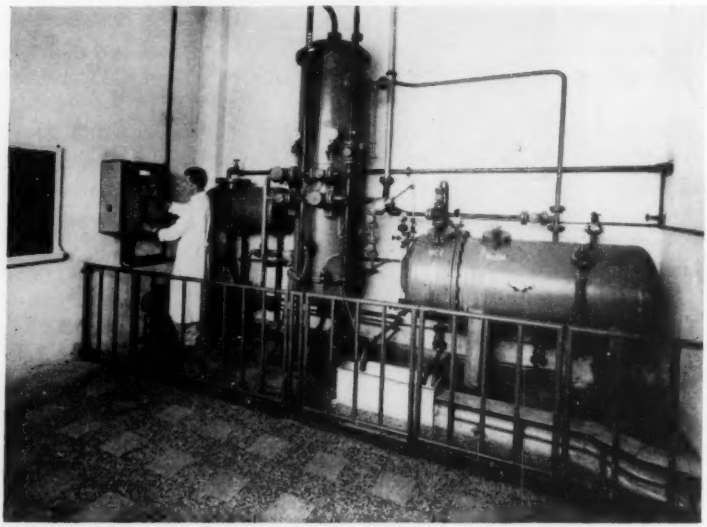


Fig. 2. De-ionizer

Automatic Cleaning Machine for the Roux Flasks

This is an ordinary industrial bottle-washing machine (Niagara 1 B) suitably modified (Fig. 3). The machine is composed of: (1) a large cylindrical-shaped drum inside which a circular trolley

runs freely, the Roux flasks to be washed being placed in the appropriate positions on the trolley, protected by rubber strips; (2) two tanks with a capacity of 400 l., one containing the detergent–sterilizer solution and the other water for the first rinsing, these liquids being recoverable and heated by steam; (3) a group of three electric pumps which drive the detergent, the water for the first rinsing and the de-ionized water for the third rinsing at a

Fig. 3. Automatic cleaning machine for Roux flasks

pressure of 2·5 atm.; these liquids are squirted through suitably spaced pipes and strike both the inside and outside of the Roux flasks. The external measurements of the machine are: 2·90 m × 2·30 m at the base, × 1·70 m high.

The soiled Roux flasks are brought into the washing-room immediately after the virus collection on the same trucks which are

used during incubation. In this way, proteinaceous deposits are prevented from drying out and this greatly facilitates cleaning. They are then placed without any previous treatment in the correct positions on the circular trolley in the washing-machine. The flasks are carried in continuous rotation successively to the four different internal sectors of the machine, where they are squirted with the various liquids for the appropriate times.

In detail, the washing of Roux flasks takes place as follows:

(1) Washing at 80°C with Neophos GM (1 per cent, recoverable) dissolved in de-ionized water, in three successive series of sprays each lasting 10 sec (total of 30 sec). This treatment has the object of rendering the Roux flask clean and killing the virus. Neophos GM with its NaOH content is a sterilizer for the F.M.D. virus.

(2) First rinsing with de-ionized water (recoverable) at 50°C in two series of sprays each lasting 10 sec (total of 20 sec).

(3) Second rinsing with tap water, not recovered, at supply temperature in eight series of sprays each lasting 10 sec (total of 80 sec).

(4) Third rinsing with de-ionized water, not recovered, at room temperature in two series of sprays each lasting 10 sec (total 20 sec).

The de-ionized water is drawn by the appropriate pump of the washing machine directly from the 4-m^3 tank mentioned above.

With this machine, up to 1,200 Roux flasks per hour can be washed.

Trucks for the Transport and Incubation of the Roux Flasks

These are trucks on rollers with 13 shelves on which 182 Roux flasks can be placed in double rows. The dimensions are: 60 cm × 88 cm × 130 cm high.

Description of the Production Cycle of a Batch of F.M.D. Virus (From 90 to 130 Litres)

The method followed is based on the classical and well-known culture technique of trypsined tissue (Youngner, 1954; Dulbecco and Vogt, 1954; Melnick 1956; Bodian 1956). Reference should be made to these works for details of preparation of the various reagents, media, etc. Here we shall describe only how the method has been adapted to industrial scale use.

The kidneys are removed from the calves as soon as they have been slaughtered, in a slaughter-house near the laboratory. For the most part the calves weigh from about 100–150 kg. These calves provide kidneys weighing about 300 g from which 100 g of cortical tissue can be recovered. The kidneys are removed in the most aseptic way possible, together with the perirenal fat, and care is taken not to damage the renal capsule in the slightest degree.

The kidneys thus removed are immediately placed in a stainless-steel recipient containing refrigerated Hanks' solution with antibiotics. Generally 8–10 calves' kidneys are removed in an afternoon (between 3 p.m. and 4 p.m.). The kidneys reach the laboratory about 30 min after slaughtering. In the laboratory three people detach first the perirenal fat, then the capsule, and with curved scissors take out the cortical tissue which is collected in large test-tubes, with a capacity of 200 ml and containing Hanks' solution. All these operations are performed in the most scrupulous conditions of asepsis. When the cortical tissue of all the kidneys has been collected in relatively large pieces, it is chopped by means of long-handled scissors until the pieces have a size of 4–5 mm. Then the tissue is washed (always adding fresh Hanks' solution, shaking and decanting it).

In this way about 800 g of tissue is ready for trypsinization and is subdivided into four to five trypsinization recipients. For this purpose flasks of pyrex glass are used, with four indentations and with a capacity of 2 l. Two hundred grams of tissue are placed in each flask and 600 ml of a sterile solution of 0·20 per cent trypsin (NBC 1:300) dosed with antibiotics (penicillin 0·07 mg/ml, streptomycin 0·1 mg/ml) are added at room temperature. To ensure that the yield of the various flasks is as homogeneous as possible, care is taken that the tissue in each flask comes from two different animals.

By means of magnetic stirrers the contents of the flasks are stirred for 2 h at about 20°C (first trypsinization). Then the supernatant is decanted and discarded. An addition is made to each flask of 1,200 ml of 0·20 per cent trypsin solution with antibiotics at refrigerator temperature. Stirring is continued overnight at a temperature of +6° to +8°C for 16–20 h (Bodian method) (second trypsinization). The following day the flasks are removed from the refrigerator, the supernatant of each is decanted

through three layers of sterile cheesecloth, into the same number of 3-l. glass flasks already containing about 1,200 ml of cold (+5°C) nutritive medium with 10 per cent calf serum. In this way the volume of the trypsinized cell suspension is doubled with this medium which, with the presence of the serum and the low temperature, inhibits the action of the trypsin during the centrifugation and at the same time dilutes the cell suspension, which at times is too dense to be deposited at the low speed of centrifugation used. At the moment two centrifuges are used with heads of 28 cm radius, holding six bottles with a capacity of about 500 ml each, in which are placed 400 ml of cell suspension. Each centrifuge has a capacity of 2,400 ml (which is the quantity of suspension to be centrifuged obtained from each flask, after doubling of the volume with nutritive medium and calf serum). Centrifuging is carried out at 800 rev/min for 20 min. Each centrifugation requires 30–40 min, if we calculate the time needed for filling the centrifuge bottles and for starting and stopping the centrifuge.

At present with two such centrifuges it is possible to centrifuge 4,800 ml of cell suspension in 30–40 min, which corresponds to the cell suspension of two flasks or of the tissue of four kidneys. The supernatant is discarded, the packed cells are suspended by means of addition of a relatively small and known quantity of nutritive medium (in general 100 ml for the six bottles of a centrifuge). The volume of packed cells is calculated by subtracting from the final volume the known quantity (100 ml) of medium used for the suspension of the sedimented cells. A yield of 50–80 ml of packed cellular material is obtained from the cortex of each kidney (which corresponds to 100–160 Roux flasks for each kidney). A sufficient quantity of concentrated cell suspension is given in large flasks containing 10 l. of sterile growth medium (Hanks' solution + 0·5 per cent lactalbumin hydrolysate + 10 per cent calf serum and antibiotics) to obtain a final dilution of 1:200 (which in general corresponds to a concentration of about 400,000 cells per ml, the count being made with crystalviolet).

During the time the cell suspension is being inoculated into the Roux flasks, the department for the preparation of cells sees to the centrifuging of the supernatant of the other flasks.

During the inoculation of the cell suspension into Roux flasks (Fig. 4), the larger flasks containing the final cell suspension and a

sterile silicone covered magnet are placed on large magnetic stirrers and are connected to the dispensing machine. Two small pumps each of 50-ml capacity, are mounted on this machine, and when connected together they enable one Roux flask to be filled (100 ml) at each revolution of the machine. The functioning of the machine is regulated at such a speed that foam and excessive pressures are avoided in the inlet and outlet valves as they might affect the vitality of the cells. Three hundred and fifty Roux flasks can be filled per hour.

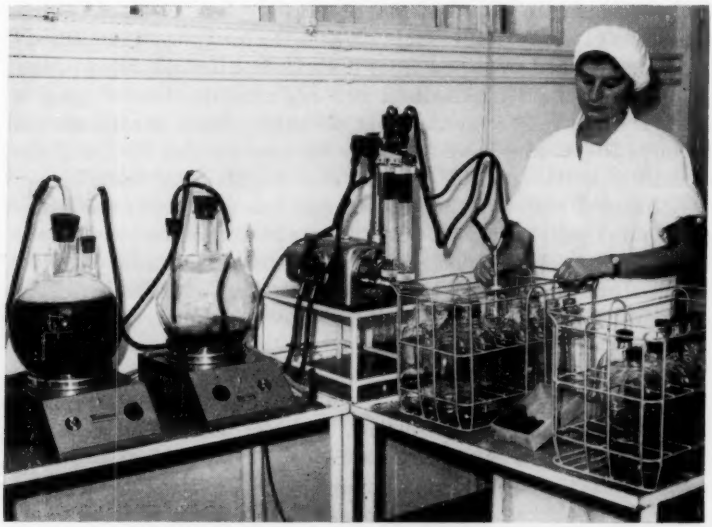


Fig. 4. Filling of cell suspension into Roux flasks

At present we use 1,000-ml Roux flasks of Hysil glass. The sterile Roux flasks, contained in special metal baskets (14 flasks per basket) are carried into room (10) for the inoculation of the cell suspension on trucks. The filling and closing of the Roux flasks is done directly in these baskets by two people; while one cuts the paper wrapping from the mouths of the flasks and fills them, the other closes the flasks with the aid of rubber stoppers with caps which fold down over the necks of the flasks. The third

person removes from the baskets those flasks which have already been filled and closed and places them on the special trucks. On these trucks the Roux flasks are put into one of the incubators for the growth of monolayers (12).

Generally, by the fifth day the development of the cellular monolayer is satisfactory and the trucks are moved to the virus inoculation room where the growth medium is taken away and replaced by 100 ml of survival medium (Hanks' solution + 0·5 per cent lactalbumin hydrolysate without serum) at room temperature. For this purpose a dispensing machine of the same type as described above is used, which we have modified so as be able to seminate the medium and virus at the same time.

The infected Roux flasks, still on the trucks, are put into the incubators (15). In a time varying from 15 to 20 h the virus multiplies, bringing about total lysis of the cellular monolayer. The virus is collected in stainless-steel vessels in the same room which is used for the virus inoculation, after which the Roux flasks, again still on the trucks, are carried to the washing machine and immediately washed.

All the operations which are necessary for the production of 400 l. of virus per week (collection of kidneys, trypsinization, cell inoculation, change of medium, inoculation of virus, collection of virus, washing of Roux flasks, sterilization, preparation of media and calf serum etc.) are performed by six laboratory workers and one technician. This production cycle is repeated three times per week.

Titrations on suckling mice of virus produced with this technique give generally a titer $10^{-7} - 10^{-7.5}$ ml.

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Mass Propagation of Algae for Photosynthetic Gas Exchange

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Summary. Engineering data on the design and operation of algal culture systems for photosynthetic gas exchange are virtually non-existent. The authors have conducted intermediate and definitive level engineering studies to characterize algal systems—with a view to extrapolating to larger-scale systems for life support in closed spaces.

Light intensity, carbon dioxide concentration, and dilution rate were the principal parameters used to control the photosynthetic rate and, consequently, oxygen production. Carbon dioxide absorption rate, equilibrium density, and cellular growth rate were also investigated.

It was found that: (1) properly jacketed high intensity, incandescent lamps provided a suitable light source for growing algae; and (2) physiologically safe (0·5 per cent) concentrations of carbon dioxide produced growth comparable to that obtained at higher concentrations.

A dilution rate of nearly 0·1 volume change per hour produced the best oxygen yield ($2{\cdot}41 \times 10^{-3}$ lb/h) for the definitive system. Maximum cell doubling time was 5·1 h. The highest culture density attained was 5·9 mm^3/ml, and the maximum dry weight algae yield was $3{\cdot}0 \times 10^{-3}$ lb/h.

Introduction

The photosynthetic process offers an attractive, workable solution to the problem of sustaining human life in a closed, or partially closed ecological system. Photosynthesis will, in a single process, fulfil three of the essential requirements of life support: it will provide a continuous supply of breathable oxygen, it will continuously remove carbon dioxide from the surrounding atmosphere, and it will yield a continuous supply of plant cells useful as food.[1]

From the standpoint of air revitalization, the problem is essentially a repetition of the Priestley experiment, or from the broader view, 'the balanced aquarium' problem. Our approach has been to repeat these experiments on a large scale in order to study the factors that influence the efficiency and stability of the cyclic system. In so doing, we have been guided by practical considerations;

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that is, we have tried to keep the bulk and weight of the algae-culture apparatus to a minimum.

The problem of designing and operating a large photosynthetic gas exchanger is a problem in the mass propagation of cells; for it is photosynthesis that controls growth rate, and oxygen will be produced at a high rate only as long as rapid growth continues.

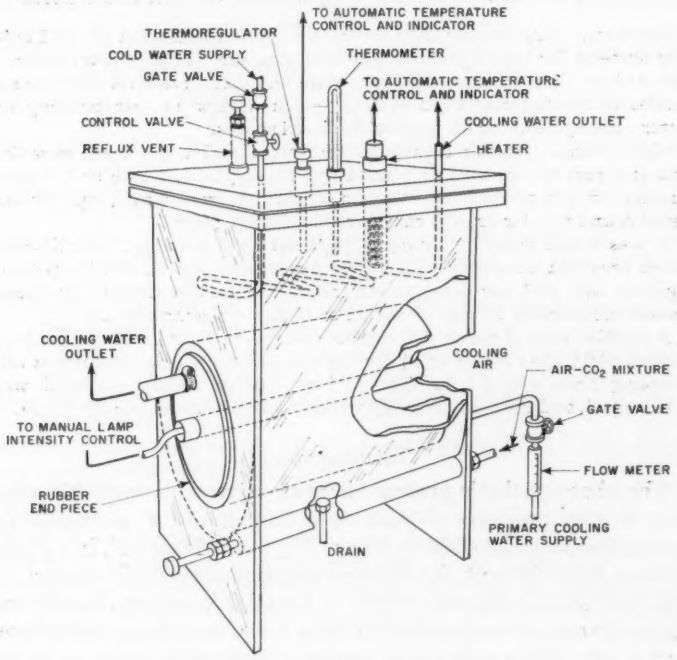

Fig. 1. Detail of 4-litre culture unit

Experiments

The basic equipment used in this study can be divided into two categories as follows:

1. Intermediate culture units of 4 litres capacity, shown schematically in Fig. 1, consisting of: (a) the culture chamber, (b) the

lighting system, (c) the primary and secondary cooling systems, (d) the heating system, (e) the combined aeration–circulation system, and (f) the automatic temperature controller–indicator.

2. The engineering-research photosynthetic gas exchanger (see Fig. 2), which consisted of the following: (a) the contact chamber (see Fig. 3), (b) the lighting system, (c) the primary and secondary cooling systems, (d) the gas exchange tower, (e) the automatic temperature controller–indicator, (f) the culture density controller–indicator, and (g) the gas analysis system.

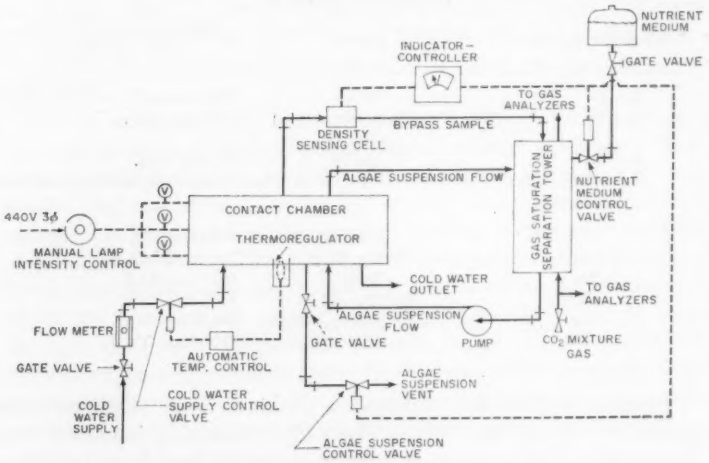

Fig. 2. Flow diagram for photosynthetic gas exchanger

Intermediate Culture Units

On the basis of previous studies,[2,3] we selected the thermophilic *Chlorella pyrenoidosa*, 71105,[4] for our experiments. The nutrient requirements of this organism have scarcely been studied since its discovery in 1951. We used a medium recommended by Dean Burk upon learning that he had obtained two-hour generation times in small-scale cultures. Three recent studies[5–7] dealing with various aspects of *Chlorella* 71105 all used a medium that differed from ours. The formula for the modified Burk's medium used in this study is shown in Table I.

Table I. Modified Burk's medium for algae culture

Basic medium

Constituent	g/l. (conc.)
$MgSO_4.7H_2O$	3·3
KH_2PO_4	1·7
NaCl	1·3

(Dissolved in tap water to make 1 litre)

Supplemental solutions

Element supplied	Source	Final concentration in basic medium
1. N	Urea	0·27g urea/l. (~0·13 g N/l.)
2. Fe	Sequestrene (NaFe)	0·37 mg Fe/l.
3. Ca	$CaCl_2$	5·3 mg Ca/l.
4. Arnon's A–5 Solution (not included when Groton tapwater is used as solvent)		
B	H_3BO_3	0·99 mg B/l.
Mn	$MnCl_2.4H_2O$	1·0 mg Mn/l.
Zn	$ZnSO_4$	0·18 mg Zn/l.
Cu	$CuSO_4.5H_2O$	0·04 mg Cu/l.
Mo	$(NH_4)_6Mo_7O_{24}.4H_2O$	0·003 mg Mo/l.
V	$Na_3VO_4.16H_2O$	0·009 mg V/l.

Inocula of *Chlorella* 71105 were obtained from stock cultures grown in 500-ml test tubes. Initially, a starting inoculum was taken from a sterile pure culture on agar obtained from Dr. Starr at Indiana University. After being transferred to a liquid mineral medium for active culturing, the organism was maintained continuously by daily transferring a small amount of it to fresh medium. In this way a supply of healthy, fast-growing algae was made available at all times. The cultures used in this study were not bacteria-free.

Cultures were supplied with mixtures of carbon dioxide and air from a system that has been previously described.[8]

The 4-litre, intermediate culture units provided simple devices for evaluating the 1,500-W Quartzline* lamp. Each intermediate unit consisted of a culture growth tank in which all the necessary

* Registered trademark of General Electric Company.

controls were immersed (see Fig. 1). The Plexiglas* tank was 13 in. × 9 in. × 7¼ in. deep and had a head space for gas between the surface of the culture liquid and the gasketed cover. A 5-in. diameter Plexiglas tube ran through the bottom of the unit. A glass tube inside this Plexiglas tube was used for mounting the 1,500-W lamp. Cooling water was circulated between these two tubes to remove most of the heat from the lamp. This arrangement left an annulus of 0·5 in. in the curved bottom half of the culture growth chamber. Circulation and turbulence were brought about by the flow of mixed gas through a row of capillary holes extending the length of the unit near the bottom. The gas then passed out of the unit through a reflux column in the cover. A manually operated transformer was used to control light intensity.

The primary cooling system consisted simply of the jacket through which a stream of water passed around the lamp. This water jacket removed the bulk of heat energy generated by the lamp. An automatic, secondary cooling coil immersed in the culture was used for fine temperature control. This coil consisted of ½-in. diameter block-tin tubing. A thermoregulator connected to an electronic relay controlled a solenoid valve which opened when the temperature rose, thus permitting cold water to pass through the coil. To permit suitable temperature control, an immersion heater was also wired to the relay and operated when the solenoid was closed. The temperature was thus maintained to within ± 0·5°C of a constant level by heating and forced cooling.

The 1,500-W incandescent lamp is 9 in. long and ⅜ in. in diameter. The lamp has a lighting efficiency of about 10 per cent at the rated voltage (277 V). The spectral distribution embraces the entire visible range, from 400 to 700 mμ, with a peak transmission in the near infrared. Since this is such a compact lamp (watt for watt) when compared to other suitable light sources, its inclusion in a photosynthetic gas exchanger design could substantially reduce the over-all volume. The highest illumination to which the algae were exposed in the intermediate unit was about 10,000 ft-cd, obtained at design voltage.

A parametric study was used to evaluate the Quartzline lamp and to obtain information on the effects of CO_2 concentration and

* Registered trademark of Rohm and Haas.

culture density. In these experiments, all runs were made in duplicate and were of 40–48 h duration. The temperature was held constant at 37 °C. The modified Burk's medium was used. During each of nine duplicate runs, the supplementary elements, iron, urea, and calcium, were added to the extent dictated by cell growth. The calculations for these additions were based on the average dry weight composition of *Chlorella* cells used by Thacker and Babcock.[9] The density of the cultures was measured every 4 h, and a suitable volume was withdrawn and replaced with fresh medium to restore density to the level set beforehand in the design. Density measurements, and consequently growth, were based on haematocrit values of small samples withdrawn from the cultures. For the design of the experiment we are indebted to the Division's Computer Section and to Dr. D. E. Nichols of the University of Rhode Island. The design is based on the analysis of variance technique as described by C. R. Hicks.[10]

The parameters studed were CO_2 concentration, culture density, and lamp voltage (light intensity), as shown in Table II(a). The experimental design tested the effect of various levels of the three parameters on growth rate. As an index of growth rate, we used the average density increase over the 40-h period, with density being read and readjusted to the preset level every 4 h.

The experimental results of the nine duplicate runs are shown in Table II(b). The growth index, R, is indicated by the last two columns of the table. The index was calculated as the change in density with time multiplied by 1,000. The over-all reproducibility in the parametric study was 0·78 on a scale of -1 to $+1$.

The experimental design enables one to determine which of the parameters involved have a significant effect over the range tested. Table II(c) shows the results of the analysis. The CO_2 level is significant at the 95 per cent confidence level, and the light intensity is highly significant at the 99 per cent level. The culture density is significant in the range studied at a confidence level of 90 per cent.

The results of this parametric study with the intermediate unit are pertinent to the design of a photosynthetic gas exchanger in several important respects. Light from an intense lamp can be utilized by the thermophilic *Chlorella*. Further, the higher the light intensity (within the limits of the lamp and unit design),

the faster is the cellular growth rate. Concentrations of CO_2 around 0·5 per cent do not appear to limit the growth of the organism. (See Test No. 9 in Table II(b).) This could conceivably permit control of the ambient CO_2 concentration in closed atmospheres at a level substantially below 1 per cent. A major breakthrough in life support systems will be realized if future studies confirm this finding.

Table II. Latin square experiment

(a) Design

Factors	Levels		
	1	2	3
% CO_2	0·5	3·0	5·5
% Cell density	0·5	1·0	1·5
Lamp voltage (relative intensity)	150 (10)	213 (38)	277 (100)

(b) Experimental results

Test no.	Levels of factors			*R* Value Unit no.	
	a	*b*	*c*	1	2
1	1	1	1	30	30
2	2	2	3	38	35
3	3	3	2	22	16
4	3	1	3	32	28
5	1	2	2	22	31
6	2	3	1	15	16
7	2	1	2	29	28
8	3	2	1	25	16
9	1	3	3	38	34

Reproducibility: 0·78 of a possible range of −1·00 to +1·00

(c) Analysis

	99%	95%	90%	% Variance accounted for*
CO_2 conc.	NS	S	S	16
Cell density	NS	NS	S	9
Light intensity	SS	SS	SS	51

* Remaining 24% variance may be ascribed to other causes.
Code: NS–Non significant, S–Significant, SS–Highly significant.

The growth rates achieved in the parametric study were about 9 h, at best, when expressed as average generation time. Since the parametric study was concluded, we have achieved a generation time of less than 4 h in the intermediate units by harvesting a portion of the culture and replacing the harvest with fresh medium at a controlled rate.

Engineering-Research Photosynthetic Gas Exchanger

The engineering-research gas exchanger design incorporated improvements based on extended operation of an earlier engineering unit developed at Electric Boat Division.[2]

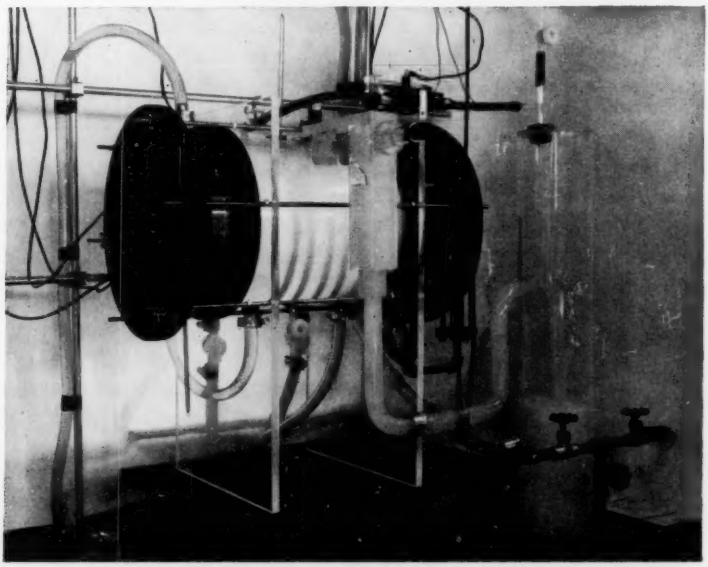

Fig. 3. Engineering-research photosynthetic gas exchanger

The gas exchanger (see Fig. 3) consisted of a light contact chamber (see Fig. 4) and a gas saturation–separation chamber. The shell of the contact chamber was constructed of a horizontally mounted, 11-in. length of 10-in. diameter Plexiglas pipe. The pipe was fitted at both ends with 1-in. thick, grooved, Buna-N rubber inner dams whose surfaces were coated with a polyamide

cured epoxy. Three cutouts in the inner dams gave press-fit support to the three horizontally mounted, outer lamp jackets. These outer lamp jackets were constructed of $2\frac{1}{2}$-in. diameter, Pyrex* tubing, and carried cooling water around the inner lamp jackets. The inner lamp jackets were constructed of 16-in. lengths of 1-in. diameter Vycor* tubing, and were press-fitted to the outer dams. The Quartzline lamps were supported on brass saddles within the inner jackets. Wooden retainer plates and six

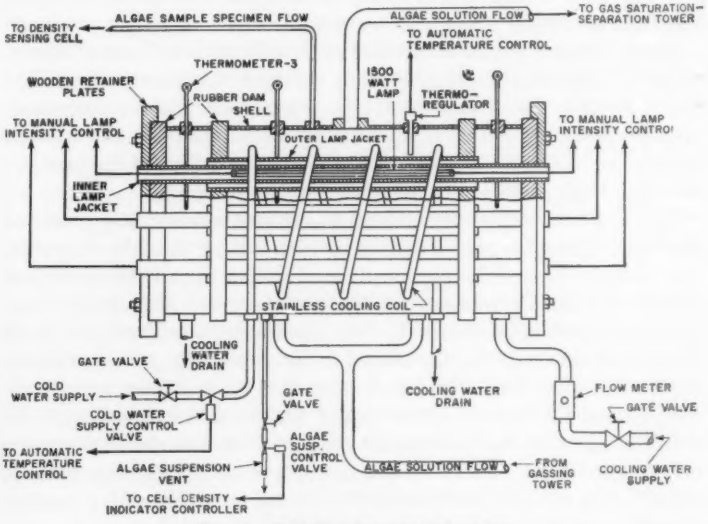

Fig. 4. Details of contact chamber

tie rods were used to hold the entire assembly together, giving watertight seals between the dams, the contact chamber, and the glass tubes.

The penetrations for the thermoregulator, thermometers, media circulating lines, water cooling lines, and drains, were made through holes in the Plexiglas shell. The dams and $2\frac{1}{2}$-in. diameter Pyrex tubes were installed for primary cooling. The flow of cooling water through this section was manually set by means of a globe valve and flowmeter. Thermometers installed at

* Registered trademark of Corning Glass.

the inlet and outlet headers were used to obtain the temperature differential. The bulk of the cooling was done by this system.

An automatically controlled secondary cooling system was used for fine temperature control of the medium. A 9-ft length of $\frac{3}{8}$-in. stainless-steel tubing was turned into an 8-in. coil and fitted into the light contact chamber. The flow of cooling water through this coil was regulated by a solenoid valve controlled by a relay which was actuated by the thermoregulator in the culture (see Fig. 2). Using this system, the temperature of the medium was held to within $\pm 0{\cdot}5$ °C of a constant level.

Lamp intensity was controlled manually with a Variac* transformer. Light intensity at various voltages was measured outside the unit and the intensity at the surface of the cooling-water jacket surrounding the lamps simply calculated by the inverse square law. Light intensities *in situ*, at the surface of the jackets, were too high to be easily and accurately measured.

The gas saturation–separation tower was a vertically oriented length of Plexiglas pipe 27 in. long with a 5-in. outside diameter. The bottom face of the pipe was fitted with a removable end piece which contained a retainer for the gas sparger. The sparger was a 316 stainless-steel disc with 165-μ porosity. A Plexiglas cover with three access ports was sealed to the top of the gas saturation–separation chamber. One port was used as a mount for a pressure gauge, the second as an outlet for the effluent gas, and the third as an inlet for the bypass sample from the density sensing cell and as an inlet for the fresh nutrient medium from the storage tank. Any of these ports could be opened to add inocula, cleaning solutions or sensing devices, as needed.

During unit operation, the aeration gas mixture first passed through a wet test meter equipped with a thermometer and a pressure gauge, and then entered from the bottom of the tower, leaving the sparger as small bubbles. Most of the gas passed up through the tower where it was scrubbed by the medium; however, a small amount of gas was drawn into the inverted intake tube from the pump. This gas was circulated with the medium through the pump, the flow meter, and connecting tubing to the illuminated contact chamber. It was then returned with the medium to a point near the top of the aeration tower where it was

* Registered trademark of General Radio Company

vented with the gas passing up through the tower. Intimate contact between the entrained gas and liquid was maintained owing to the turbulent flow created by many bends in the circulating lines.

The culture was circulated between the contact chamber and the aeration tower by a variable speed gear pump. The medium entered the light contact chamber tangentially from both ends. In the chamber, the medium swirled around the glass lamp jackets and stainless-steel coils of the heat exchanger, and left at the top centre through a tangential fitting. It then passed through a U-trap back into the tower where it streamed out to give a large liquid surface area for release of the entrained gas.

The gas analysis system consisted of an indicating paramagnetic oxygen analyzer and an indicating infrared CO_2 analyzer. Both of these instruments were calibrated daily.

Before start-up, the gas exchanger was cleaned with a solution of calcium hypochlorite and thoroughly rinsed. The nutrient medium was added and heated to the desired operating temperature. Inocula from the stock cultures were then added and the lamps operated.

The unit was run on a continuous basis using manual control. During a run, hourly readings were made on the oxygen and CO_2 content of the inlet and outlet gas, the influent gas flow rate, influent gas temperature, influent gas pressure, barometric pressure, ambient temperature, media temperature, coolant inlet and outlet temperatures, coolant flow rate, and culture flow rate. Several other readings were also taken as a check on the physical condition of the system. In addition, a sample of the culture was taken for the determination of pH and optical density. Density measurements were made spectrophotometrically. Actual cell counts and packed cell volume were taken periodically as a check on the density readings.

The Factorial Experiment

Three preliminary runs were required to establish the levels of the variables used in the factorial design. Including preliminary runs 1–3, the experiment was conducted on a single, continuously-operated culture over a period of 14 days. Three levels of light intensity were combined with three rates of dilution as outlined in Table III, which shows three additional runs, 13–15.

Table III. Factorial experiment with engineering-research photosynthetic gas exchanger

Run no.	Light intensity (ft-cd)	Power input (kW)	Dilution rate (l./h)	O_2 production (lb/h)	O_2 production (ft^3 at 70°F/h)	Equilibrium density (mm^3/ml)	Wet algae produced (lb/h)	Dry weight algae produced (lb/h)	CO_2 absorption rate (lb/h)	Assimilatory quotient (Mole CO_2/ Mole O_2)
4	15,000	1·53	1	$1·72 \times 10^{-3}$	$1·93 \times 10^{-2}$	3·26	$7·21 \times 10^{-3}$	$1·8 \times 10^{-3}$	$2·15 \times 10^{-3}$	0·92
5	25,000	2·04	1	$2·01 \times 10^{-3}$	$2·26 \times 10^{-2}$	3·71	$8·11 \times 10^{-3}$	$2·0 \times 10^{-3}$	$2·97 \times 10^{-3}$	1·08
6	35,000	2·57	1	$2·41 \times 10^{-3}$	$2·71 \times 10^{-2}$	4·30	$9·51 \times 10^{-3}$	$2·4 \times 10^{-3}$	$2·95 \times 10^{-3}$	0·90
7	25,000	2·04	0·5	$1·52 \times 10^{-3}$	$1·71 \times 10^{-2}$	5·87	$12·0 \times 10^{-3}$	$3·0 \times 10^{-3}$	$2·64 \times 10^{-3}$	1·25
8	25,000	2·04	1·5	$1·79 \times 10^{-3}$	$2·01 \times 10^{-2}$	2·42	$5·34 \times 10^{-3}$	$1·3 \times 10^{-3}$	$2·75 \times 10^{-3}$	1·11
9	35,000	2·57	1·5	$1·91 \times 10^{-3}$	$2·15 \times 10^{-2}$	2·18	$4·82 \times 10^{-3}$	$1·2 \times 10^{-3}$	$2·95 \times 10^{-3}$	0·96
10	15,000	1·53	1·5	$1·36 \times 10^{-3}$	$1·53 \times 10^{-2}$	2·26	$4·99 \times 10^{-3}$	$1·3 \times 10^{-3}$	$1·72 \times 10^{-3}$	0·92
11	35,000	2·57	0·5	$2·10 \times 10^{-3}$	$2·36 \times 10^{-2}$	4·26	$9·40 \times 10^{-3}$	$2·3 \times 10^{-3}$	$3·20 \times 10^{-3}$	1·01
12	15,000	1·53	0·5	$1·22 \times 10^{-3}$	$1·37 \times 10^{-2}$	4·38	$9·65 \times 10^{-3}$	$2·4 \times 10^{-3}$	$1·79 \times 10^{-3}$	1·06
13	42,000	3·07	1·0	$1·87 \times 10^{-3}$	$2·10 \times 10^{-2}$	4·02	$8·86 \times 10^{-3}$	$2·2 \times 10^{-3}$	$2·32 \times 10^{-3}$	0·88
14	55,000	4·05	1·0	$1·20 \times 10^{-3}$	$1·35 \times 10^{-2}$	3·38	$7·46 \times 10^{-3}$	$1·9 \times 10^{-3}$	$1·95 \times 10^{-3}$	1·19
15	60,000	4·50	1·0	$1·01 \times 10^{-3}$	$1·14 \times 10^{-2}$	2·83	$6·26 \times 10^{-3}$	$1·5 \times 10^{-3}$	$1·23 \times 10^{-3}$	0·91

Temperature was held at 39° ±0·5°C. The culture was supplied with 3·5 per cent CO_2 in air at a rate of 2·0 ±0·2 cubic feet per hour. The total pressure on the system was held at 15·3 lb/in² ±0·4 lb/in². Algal suspension was circulated at a rate of 7 g/min (26·5 l./min). A total volume of 11 l. of suspension was maintained in each run. In each run, the culture was diluted with complete tap water medium at the given rate, and was permitted to reach an equilibrium density. Density was measured spectrophotometrically every hour.

Gas measurements were made hourly throughout the entire experiment but only values taken during equilibrium were used in the factorial design. The averages of the four values of O_2 production and CO_2 absorption were taken as definitive for each run. It should be emphasized that equilibrium density, CO_2 absorption rate, and O_2 production rate are dependent variables.

Equilibrium densities for the 12 runs are given in column 7 of Table III. Note that the maximum density was obtained at intermediate light intensity and lowest dilution rate (run no. 7 at 25,000 ft-cd, 0·5 l./h). The last three rows in Table III are from an extension of the factorial experiment (runs 13, 14, 15) in which the effect of very high light intensity was assessed. Growth was markedly inhibited at these higher intensities, confirming the results of Burk who indicated that solarization of *Chlorella* occurs below 35,000 ft-cd in flashing light.[11] Although steady illumination was used, the circulation pattern imposed conditions equivalent to intermittent light.

Oxygen production, as a function of light intensity (power input) and dilution factor, is shown in the three-dimensional plot in Fig. 5. The surface shows that maximum oxygen production occurs in the region of 35,000 ft-cd and 1·0 l./h dilution rate (0·09 dilution factor*). The surface also shows that for all light intensities in the factorial design, highest oxygen production was obtained at the middle dilution rate. The solarization effect above 35,000 ft-cd is shown dramatically by the rapid fall in oxygen production. In fact, the solarization effect is more immediately pronounced on oxygen production than on equilibrium density, as is shown in Fig. 6.

* Dilution factor is the dilution rate in l./h divided by the volume of the system in litres; it has the units of reciprocal hours.

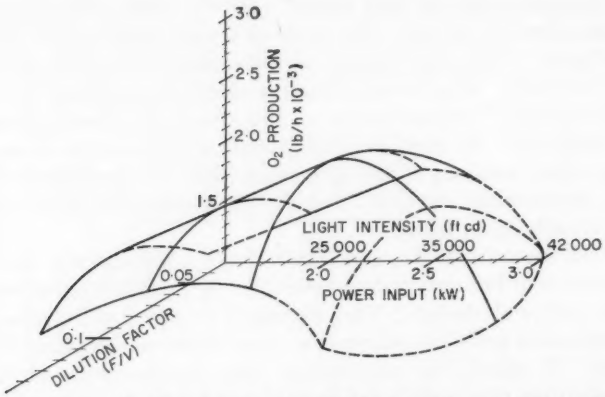

Fig. 5. Oxygen production as a function of light intensity and dilution factor

It should be emphasized that the maximum region observed is characteristic of the actual gas exchanger used in these studies. Another salient feature of the surface is that it shows the profound effect of dilution factor on oxygen production.

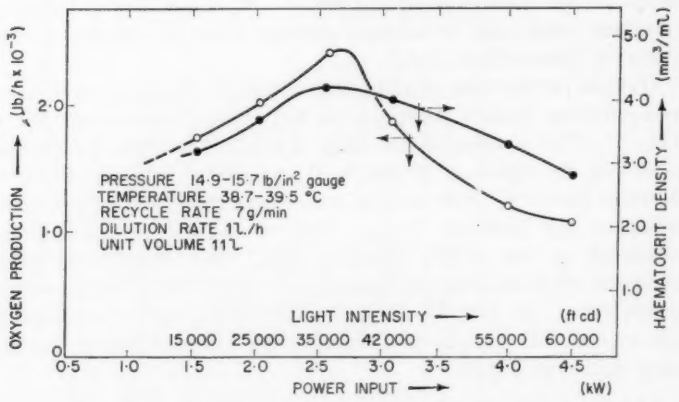

Fig. 6. Oxygen production and equilibrium density as a function of light density

The rate of cellular growth is another measure of the response of the organisms to the environmental conditions. Growth rate can be expressed as the specific generation time or as the cell doubling time. In terms of actual yield in a steady-state culture, the specific generation time (t_s = fixed volume/dilution rate) of the overall system is the more meaningful. The specific generation times at different dilution rates are shown in column 2 of Table IV. At equilibrium density, the generation time is inversely related to the dilution rate over the limits tested. The cell doubling time (t_d) is derived in the following way.[12]

Table IV. Culture growth rates at equilibrium density for a system with 11 litres total volume

Dil. rate (l./h)	Specific generation time (h)	Cell doubling time (h)	Growth rate (cell div./day)	Equilibrium density (mm^3/ml)
0·5	22·0	15·2	1·6	5·9
1·0	11·0	7·6	3·2	4·3
1·5	7·3	5·1	4·7	2·4

Let the growth rate and characteristic of the species and culture system be fixed. Then, the rate at which the number of cells in the system will be increasing (i.e., the density) will be proportional to the number of cells present, $dN/dt = kN$, or $dN/N = kdt$. Integration gives: $\ln N = kt + c$, when $t = 0$ and $c = \ln N_0$. Therefore, $\ln N - \ln N_0 = kt$, or $\ln (N/N_0) = kt$. For t equal to the doubling time, $N/N_0 = 2$; therefore, $t_d = \ln 2/k$, where k = dilution rate/fixed volume. It follows, therefore, that $t_d = 0{\cdot}693 \times$ fixed volume/dilution rate, or $0{\cdot}693t_s$.

In other words, the so-called doubling rate will always be simply less than the specific generation time by a factor of about 0·7, and consequently, it is also inversely related to dilution rate.

It is important to note that the dilution rate (1·0 l./h) which gave the maximum oxygen production (Fig. 5) did not result in the highest growth rate (lowest generation time) as is shown in

Table IV. This can be most simply explained by the fact that, although growth rate was highest at the highest dilution rate (1·5 l./h), the equilibrium density was not high enough to support photosynthesis equal to that obtained at 1·0 l./h and at a higher equilibrium density.

Directly related to equilibrium density is algae production. Column 8 of Table III shows the average production of algae in pounds fresh weight per hour during the equilibrium period of each run. As might be expected, high yield of algae generally occurred with high yield of O_2. A series of dry weight determinations on algae paste from the centrifuge gave a moisture content of 76 per cent. The wet-weight figures can be converted to dry weight by simply dividing by four. There are a number of ways of determining the moisture content of the material, and pastes prepared in different ways will yield different results; however, the value of 25 per cent dry weight has been widely used. The dry weight figures thus obtained (column 9, Table III) indicate that the synoptic expression for photosynthesis held reasonably well (dry weight of cells produced approximately equal to weight of oxygen produced) during runs 4, 5, 6, 10, 11, and 13. There is strong disagreement between the two values in runs 7 and 12. Agreement is poor in runs 8, 9, 14, and 15. On a qualitative basis, the discrepancies can be accounted for by variations in the density and activity of bacterial contaminants in the system.

Analysis of Variance

The data of the factorial experiment were subjected to analysis of variance. The four equilibrium values of the dependent variables (density, O_2 production) in each run were treated as replicates. The independent variables were power input and dilution rate. The analysis showed:

1. Oxygen production is significantly affected (95 per cent confidence level) by both power input and dilution rate as compared to the experimental error. The interaction of power input and dilution rate proved insignificant.

2. Equilibrium density is significantly affected by power input and by dilution factor. Furthermore, there is a significant interaction between the independent variables in their effect on equilibrium density.

Discussion

The results of the experiments described above are significant both with regard to the behaviour of mass cultures of microscopic algae and to the characteristics of a photosynthetic gas exchange apparatus. Certain features for future designs of engineering models are suggested by the results.

Oxygen Production

Two primary features of the system are of major importance. The first is the maximum oxygen production rate; the second, the oxygen production per unit of power input, which is one way of expressing the efficiency of the system. Cell production can be related to oxygen production or efficiency by reference to Table III.

The maximum rate of O_2 production ($2{\cdot}41 \times 10^{-3}$ lb O_2/h) was observed at a power input of 2·57 kW and a dilution rate of 1 l./h and at an equilibrium density of 4·30 mm³/ml (0·43 per cent v/v). This production occurred at an efficiency (O_2 production/power input $= 0{\cdot}93 \times 10^{-3}$ lb/h kW) close to the maximum efficiency observed ($1{\cdot}12 \times 10^{-3}$). It should be recalled here that this maximum production and high efficiency occurred at a light intensity of 35,000 ft-cd. In order to keep the surface intensity at 35,000 ft-cd and to preclude gross solarization, it was necessary to operate at only 195 V. This lowered the efficiency of the 277-V lamps from 10 per cent to about 5 per cent. The O_2 output could undoubtedly be increased significantly by increasing the size of the luminous surface in the apparatus. This could be accomplished simply by increasing the size of the water jackets around the lamp tubes and operating at increased voltage. As used in the factorial study, the unit had water jackets of a 2·5-in. diameter. In order to keep the surface intensity at 35,000 ft-cd at a voltage of 277 V, it would be necessary to increase the diameter of the water jackets to 3·25 in. This could be accomplished without increasing the over-all size of the unit and would increase the radiant energy output by a factor of three.

Carbon Dioxide Utilization Rate: Assimilatory Quotient

Average CO_2 utilization rates during the equilibrium periods are shown in column 10 of Table III. In column 11 are shown the values of the assimilatory quotients (AQ). The assimilatory

quotient is a standard parameter for photosynthesis; it corresponds to the respiratory quotient (RQ) in man. RQ is the molar ratio of CO_2 produced to O_2 absorbed in respiration. AQ is defined as the molar ratio of CO_2 absorbed to O_2 produced in photosynthesis. The synoptic equations for photosynthesis (1) and respiration (2):

$$6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2 \quad (1)$$

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \quad (2)$$

suggest that these quotients AQ and RQ should have a value of precisely 1·0. Since glucose ($C_6H_{12}O_6$) is rapidly converted to fats, proteins, and other constituents of protoplasm in the over-all assimilatory metabolism of green plants, measured values of the AQ deviate from this value of 1, being generally less than one. The source of nitrogen available to the plant affects the AQ value as shown by Doney and Myers[13] and others. Also, since fats and proteins are in part the substrate for catabolism in man along with glucose, RQ values for man are lower than 1·0. The fact that the end-product of protein break-down in man is largely urea also affects the value of the RQ. For man, a good average value of the respiratory quotient is 0·82; Doney and Myers found that they could very nearly match this value when their *Chlorella* cultures were fed urea, but not at all when nitrogen was supplied by the nitrate. In the factorial experiment, the AQ value was consistently higher than 0·82, averaging 1·01 even though the culture was supplied with nitrogen only in the form of urea. There are several explanations for this happening:

1. The presence of variable numbers of bacteria in the culture would have a complexing effect on the AQ of the system.

2. It is known that algae secrete organic acids and other organic moieties into the culture medium. It is, in fact, on these secreted metabolites that the guest heterotropic bacteria grow.

3. The third possibility is one which could only increase the AQ to a value higher than the 'true' value of 0·82. It is known that CO_2 can be absorbed by algae in the dark and stored until photolysis of water (the O_2 yielding reaction) can provide a reductant for the assimilation of the CO_2 condensation product. Naturally this 'debt' cannot go on indefinitely, but eventually must be paid off by photolysis, yielding oxygen. Apparently the

debt can persist for as long as the 4-h periods during which the culture was maintained at the equilibrium densities.

The very high values (1·25, 1·19) during runs 7 and 14, respectively, require special comment. Nothing in the protocol or data records suggests that these values are spurious. In any case, there is a mechanism that could account for the values in a qualitative manner. Recently it has been shown that amino acids and low molecular weight peptides can form complexes with CO_2 in the presence of calcium and perhaps magnesium.[14] During run 7, a high density persisted under the minimal dilution rate. Under these conditions one would expect metabolites to build up in the medium. If proteins were actively undergoing hydrolysis, the presence of amino acids and peptides in the medium would provide a mechanism for high CO_2 utilization. It should be recalled also that high CO_2 utilization (without concomitant O_2 production) will *raise* the AQ value. CO_2 production without O_2 absorption, as in a burst of fermentative activity on the part of bacterial contaminants, would *lower* the overall AQ value, not raise it.

Stability

Culture stability is intimately related to the medium replacement regime. Active cultures in which the medium is replaced at least twice a day remain relatively stable. A pH of 5·5 to 6·0 is maintained and periodic bacterial plate counts are as low as 120,000 cells per ml and always less than 1 million cells per ml. This can be contrasted with a week-old culture in which only a fifth of the medium had been replaced daily. In this culture, the pH was around 8·0 and bacterial count around 400 million cells per ml. Operation of a photosynthetic gas exchanger with a short replacement time obviates such difficulties.

Culture stability is also lessened by poor temperature control. Whenever the temperature exceeds the optimum for even a short period, injury to the cells is evidenced by a decrease in growth rate, foaming, and by the settling and clumping of cells. For these reasons, stable temperatures near the optimum value must be carefully maintained. Low temperatures must be avoided as well, especially at high light intensity. At low temperature, solarization and growth inhibition occur at lower light intensity than at the temperature optimum.

Conclusions

Intermediate Unit—Parametric Study

In a laboratory photosynthesis unit, it was determined that *Chlorella* cultures grew as well at low CO_2 concentrations (0·5 per cent) as they did at higher concentrations. If this is confirmed in scaled-up units, we may conclude that a photosynthetic gas exchanger can be operated on circulated cabin air maintaining physiologically safe CO_2 tension.

Very high numbers of non-pathogenic aerobic bacteria developed in the intermediate-scale cultures unless the cultures were diluted at a high rate. The dilution rate of cultures on any scale cannot be increased indefinitely, of course, and the optimum dilution rate for algae growth and O_2 production may occasionally permit high bacterial populations to build up. The feasibility of pure-culture operation of the photosynthetic gas exchanger has yet to be determined.

Engineering-Research Unit

Operation of the engineering model gas exchanger disclosed that the three General Electric 1,500-W Quartzline lamps could not be operated at design voltage if solarization of the thermophilic *Chlorella* was to be avoided. Culturing at 195 V gave an optimum intensity of 35,000 ft-cd. To obtain equilibrium growth, with maximum oxygen production, a dilution factor of 0·09 per hour was required.

Equipment design modifications, including a cooling water jacket 3·25 in. in diameter, should permit operation at design voltage, thus increasing the lamp efficiency from 5 to 10 per cent or higher and triple the amount of radiant energy available for photosynthesis. Running the lamp at still higher voltages would increase the percentage of radiation in the visible range giving even higher efficiency. This, of course, would shorten the life of the lamp which is rated at better than 2,000 h.

The maximum oxygen production of a steady-state system does not necessarily occur at the maximum equilibrium cell density, nor at the maximum growth rate. At high densities, there can be an accumulation of photosynthetically inactive algal cells and of algal metabolites which support the growth of aerobic (oxygen

using) heterotrophic bacteria. At maximum growth, the equilibrium density is not high enough to support photosynthesis equal to that obtained at a higher density and correspondingly longer doubling time. Solarization was shown to give a disproportionally large drop in oxygen production in contrast to the rate of decline of the equilibrium culture density.

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Studies on the Fermentative Production of L-Isoleucine*

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Summary. Several strains of *Pseudomonas* were selected by the screening test for organisms which are capable of producing L-isoleucine.

With these selected bacteria, various cultural conditions suitable for the production and accumulation of L-isoleucine were investigated. As a result, 12–14 mg/ml of L-isoleucine was formed by shake culture in a medium containing α-aminobutyric acid, glucose, cornsteep liquor, urea and inorganic salts.

Introduction

With recent progress in the nutritional chemistry of amino acids, the significance of the rôle played by L-isoleucine becomes clear, and an economical supply of this essential amino acid is earnestly wanted in the pharmaceutical field and for food enrichment.

However, there has been no useful method for the production of L-isoleucine until now. Protein hydrolyzates are a possible source of this amino acid but the proportion of L-isoleucine is usually low, and isolation in pure form, especially separation from leucine, requires difficult procedures.

There have also been described a number of synthetic methods for isoleucine, but the products are usually composed of four stereoisomers of isoleucine and a troublesome resolution procedure is required to obtain L-isoleucine, the only isomer available for human nutrition.

Meanwhile, a very efficient procedure, 'amino acid fermentation', has been successfully applied to the production of amino acids such as glutamic acid and lysine. On the fermentative production of L-isoleucine, however, the only available report,[1] is the formation of about 6 mg/ml in a medium containing α-aminobutyric acid with the bacteria belonging to genus *Bacillus*.

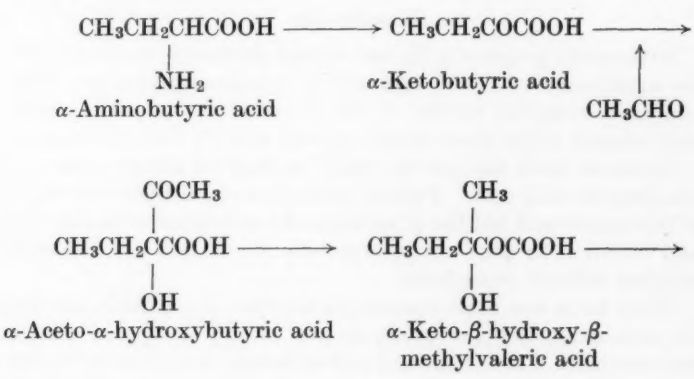
* Presented at the 1st Symposium on Amino Acid Fermentation, Kyoto, November 4, 1959.

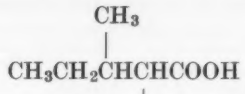
Independently, during the course of the studies on the fermentative production of amino acids of the aspartic family, the present authors found that several strains of the genus *Pseudomonas* are capable of forming and accumulating a large amount of L-isoleucine in a medium containing DL-α-aminobutyric acid. The latter is readily available by a synthetic procedure.[2,3]

Employing the selected bacteria, cultural conditions suitable for production of L-isoleucine were investigated, and levels of up to 12–14 mg/ml were obtained under the best conditions.

By the further development of the present study on a large scale, this fermentative procedure is likely to become one of the most useful procedures for the production of L-isoleucine.

According to the present knowledge of biosynthetic pathways of L-isoleucine, formation from α-aminobutyric acid is supposed to proceed as follows:





Isoleucine

However, it is not certain whether or not α-aminobutyric acid acts as direct precursor as shown in the above scheme. Consequently, conclusive explanations of the rôle of α-aminobutyric acid in the accumulation of L-isoleucine has to await further investigation.

Experimental Procedure

Organisms. Fifty strains of bacteria, five strains of streptomyces and five strains of moulds were used for the screening test. These organisms were supplied by the Institute of Applied Microbiology of Tokyo University, the Department of Fermentation Technology of Osaka University, and the Research Institute for Microbiological Diseases of Osaka University.

Media. Compositions of the culture media employed for the experiments are shown in Table I. Unless otherwise noted, the pH of the medium was adjusted to 7·0.

Screening experiments. Slant cultures of tested organisms were carried out on an agar medium containing 1 per cent peptone, meat extract and yeast extract for 1–3 days in the case of bacteria and streptomyces, and on an agar medium of malt extract (10° Bg.) for 2–4 days in the case of moulds. The temperature was 30°C.

Media S–1 and S–2 were distributed in 3-ml amounts to test-tubes and then sterilized. After inoculation with a loopful of test organisms from the slant cultures, shaking culture was carried out for 3–8 days at 30°C.

Routine identification and rough quantitative estimation of the formed amino acids were made by paper chromatography of the cultural broth. Paper chromatography was carried out with the ascending method using Tôyô filter paper No. 51 and butanol: acetic acid : water, 4 : 1 : 1, as solvent. After development the

Table I. Composition of media

Medium No.	α-Amino-butyric acid (%)	Glucose (%)	Ammonia source	(%)	Organic nitrogen source	(%)	Aspartic acid (%)
S–1	1	5	$(NH_2)_2CO$	1	Peptone	0·2	—
					Yeast extract	0·35	
S–2	1	1	$(NH_2)_2CO$	0·1			3
AB–1	1	0	$(NH_2)_2CO$	0·5			—
AB–2	1	1	$(NH_2)_2CO$	0·5			—
AB–3	1	5	$(NH_2)_2CO$	0·5			—
AB–4	1	10	$(NH_2)_2CO$	0·5			—
AB–5	1	15	$(NH_2)_2CO$	0·5			—
AB–6	1	20	$(NH_2)_2CO$	0·5			—
AB–7	1	0	$(NH_2)_2CO$	0·5			1
AB–8	1	1	$(NH_2)_2CO$	0·5			1
AB–9	1	5	$(NH_2)_2CO$	0·5			1
AB–10	1	10	$(NH_2)_2CO$	0·5			1
AB–11	1	15	$(NH_2)_2CO$	0·5	Peptone	0·2	1
AB–12	1	20	$(NH_2)_2CO$	0·5	Yeast Extract	0·35	1
AB–13	1	10	—				—
AB–14	1	10	$(NH_2)_2CO$	1			—
AB–15	1	10	$(NH_2)_2CO$	2			—
AB–16	1	10	$(NH_2)_2CO$	3			—
AB–17	1	10	$(NH_2)_2CO$	5			—
AB–18	2	10	$(NH_2)_2CO$	1			—
AB–19	2	10	$(NH_2)_2CO$	2			—
AB–20	2	10	$(NH_2)_2CO$	3			—
AB–21	1	10	NH_4Cl	1·8			—
AB–22	1	10	$(NH_4)_2HPO_4$	2·2			—
AB–23	1	10	NH_4NO_3	2·6			—
AB–24	1	10	$(NH_4)_2SO_4$	2·2			—
AB–25	1	10	$(NH_2)_2CO$	1	Casein hyd.	1·1	—
AB–26	1	10	$(NH_2)_2CO$	1	Cornsteep liquor	2·2	—
AB–27	1	10	$(NH_2)_2CO$	1	Peptone	0·9	—
AB–28	1	10	$(NH_2)_2CO$	1	Yeast extract	0·9	—
AB–29	1	10	$(NH_2)_2CO$	1	Meat extract	7·1	—
AB–30	1	10	$(NH_2)_2CO$	1	Peptone	0·45	—
					Yeast extract	0·45	—
AB–31	1	10	$(NH_2)_2CO$	1	Peptone	0·45	—
					Meat extract	3·5	—
AB–32	1	10	$(NH_2)_2CO$	1	Soybean meal	1·5	—
AB–33	1	10	$(NH_2)_2CO$	1	Gelatin hyd.	1·1	—
AB–34	1	15	$(NH_2)_2CO$	1·5	Peptone	0·9	—
AB–35	1	15	$(NH_2)_2CO$	1·5	Cornsteep liquor	2·2	—
AB–36	2	15	$(NH_2)_2CO$	1·5	Peptone	0·9	—
AB–37	2	15	$(NH_2)_2CO$	1·5	Cornsteep liquor	2·2	—
AB–38	3	15	$(NH_2)_2CO$	1·5	Peptone	0·9	—
AB–39	3	15	$(NH_2)_2CO$	1·5	Cornsteep liquor	2·2	—

Table I. *Continued*

AB–40	2	10	$(NH_2)_2CO$	1·5	Cornsteep liquor 0·55	—
AB–41	2	10	$(NH_2)_2CO$	1·5	Cornsteep liquor 1·1	—
AB–42	2	10	$(NH_2)_2CO$	1·5	Cornsteep liquor 2·2	—
AB–43	2	10	$(NH_2)_2CO$	1·5	Cornsteep liquor 4·4	—
AB–44	3	10	$(NH_2)_2CO$	1·5	Cornsteep liquor 0·55	—
AB–45	3	10	$(NH_2)_2CO$	1·5	Cornsteep liquor 1·1	—
AB–46	3	10	$(NH_2)_2CO$	1·5	Cornsteep liquor 2·2	—
AB–47	3	10	$(NH_2)_2CO$	1·5	Cornsteep liquor 4·4	—
AB–48	2	15	$(NH_2)_2CO$	1·5	Cornsteep liquor 0·55	—
AB–49	2	15	$(NH_2)_2CO$	1·5	Cornsteep liquor 1·1	—
AB–50	2	15	$(NH_2)_2CO$	1·5	Cornsteep liquor 4·4	—
AB–51	3	15	$(NH_2)_2CO$	1·5	Cornsteep liquor 0·55	—
AB–52	3	15	$(NH_2)_2CO$	1·5	Cornsteep liquor 1·1	—
AB–53	3	15	$(NH_2)_2CO$	1·5	Cornsteep liquor 4·4	—

Besides the above constituents, all media contain 0·1% K_2HPO_4, 0·05% $MgSO_4 \cdot 7H_2O$ and 1% $CaCO_3$ (2% in medium S—1).

chromatograms were sprayed with 0·2 per cent ninhydrin in water-saturated butanol, and colour development of the spots was measured.

Fermentation experiments. Fermentation experiments were carried out with the organisms selected by the screening experiments.

The respective media were distributed in 3-ml amounts to test-tubes or in 50-ml amounts to 500-ml shaking flasks, sterilized and inoculated with a loopful or with four loopfuls of inoculum, respectively. The cultures were incubated at 30° or 37 °C in a reciprocating shaker (90, 100, 110 c/min with an 8-cm stroke) or without shaking.

Of the fermentation experiments on the cultural conditions for L-isoleucine accumulation, (1)–(6) were carried out in test tubes and (7)–(9) were performed in shaking flasks.

Analysis of the culture broth was carried out as described below.

Analytical Methods. The assay of the supernatant solution from the culture broth for L-isoleucine was carried out by ordinary microbioassay[4] with the organism *Leuconostoc mesenteroides* P–60. Paper chromatography was also used for rough quantitative estimation of the amino acids formed and the α-aminobutyric acid consumed.

Quantitative determination of glucose was performed by the method of Fehling–Lehmann–Schoorl.[5]

Results and Discussion

Screening Experiments on Organisms

As a result of the screening experiments carried out by the method described above, accumulation at a level of 2–4 mg/ml in the screening media was found among strains of *Pseudomonas* bacteria. Besides *Pseudomonas* bacteria, several strains of *Escherichia* and *Serratia* showed lower degrees of L-isoleucine accumulation. However no, or very little, accumulation was observed with streptomyces and moulds under the conditions employed here.

From the above experiments, *Pesudomonas aeruginosa* NK and MO, and *Pseudomonas fluorescens* 6009–2 were selected as the most favourable strains and were employed for the following fermentation experiments.

On the Cultural Conditions for Formation and Accumulation of L-*Isoleucine*

1. *Effects of glucose concentration and addition of aspartic acid.* The effect of glucose concentration was studied with media AB–1 to AB–6 which contain 1 per cent α-ABA (abbreviation for α-aminobutyric acid) and varied amounts of glucose (0–20 per cent).

As shown in Table II, formation of 4–5 mg/ml of isoleucine was observed at glucose concentrations of 5–15 per cent.

In 0 and 1 per cent glucose, accumulation of isoleucine was not observed, although the consumption of α-ABA was not very different. The small formation of L-isoleucine at lower concentrations of glucose may be due to the lack of acetaldehyde, a metabolite of glucose, and required for the biosynthesis of isoleucine.

On the other hand, higher concentration of glucose, such as 20 per cent, also showed lower formation of isoleucine, which may be the result of growth retardation of the organisms.

According to the present knowledge of the synthetic pathways of isoleucine in organisms, aspartic acid is a precursor, and it is also possible that aspartic acid plays the rôle of amino donor in the formation of isoleucine from α-ketoisoleucine by a transamination reaction. Thus with the addition of aspartic acid, the concentration of glucose was varied in media AB–7 to AB–12, and its influence on the accumulation of isoleucine was examined. The results obtained are shown in Table III.

Table II. Effect of glucose concentration on the accumulation of L-isoleucine

Medium	Glucose %	Strain*	L-Isoleucine formed (mg/ml) Days of culture		
			2	4	6
AB–1	0	Ps. a. NK	0	0	0·5
		Ps. a. MO	0	0	0·5
		Ps. f.	0	0	0
AB–2	1	Ps. a. NK	0	0	0·5
		Ps. a. MO	0·5	0·5	1
		Ps. f.	0	0	0·5
AB–3	5	Ps. a. NK	0·5	0·5	1
		Ps. a. MO	1	1	1
		Ps. f.	1	4	1
AB–4	10	Ps. a. NK	1	3	2
		Ps. a. MO	1	2	1
		Ps. f.	1	4	2
AB–5	15	Ps. a. NK	2	2	5
		Ps. a. MO	2	2	3
		Ps. f.	1	2	3
AB–6	20	Ps. a. NK	1	2	2
		Ps. a. MO	1	2	2
		Ps. f.	0	1·5	2

Shaking culture (100 c/min) was carried out at 30°.

* Ps. a. NK, Ps. a. MO and Ps. f. represent *Pseudomonas aeruginosa* NK, MO and *Pseudomonas fluorescens* 6009–2 respectively.

Comparing Tables II and III, no marked difference is observed in the amounts of isoleucine formed with or without addition of aspartic acid. With *Pseudomonas fluorescens* 6009–2, addition of aspartic acid retarded isoleucine formation, and in general its addition resulted in a tendency to form other amino acids such as glutamic acid and alanine.

2. *Effect of urea concentration.* In order to find out a suitable ratio of glucose and ammonia source for isoleucine formation, effect of urea concentration was studied with media AB–4 and AB–13 to AB–20. The results are shown in Table IV.

Table III. Effects of addition of aspartic acid and of glucose concentration on the accumulation of L-isoleucine

Medium	Glucose (%)	Strain	L-Isoleucine formed (mg/ml) Days of culture		
			2	4	6
AB–7	0	Ps. a. NK	0	0	0
		Ps. a. MO	0·5	0·5	0
		Ps. f.	0	0	0
AB–8	1	Ps. a. NK	0	0	0·5
		Ps. a. MO	2	1	1
		Ps. f.	0	0	0
AB–9	5	Ps. a. NK	1	0·5	0·5
		Ps. a. MO	2	2	4
		Ps. f.	1	0·5	0·5
AB–10	10	Ps. a. NK	1·5	3	4
		Ps. a. MO	2	2	1
		Ps. f.	0·5	1	1
AB–11	15	Ps. a. NK	1	2·5	2·5
		Ps. a. MO	1·5	2·5	2·5
		Ps. f.	1	1	1
AB–12	20	Ps. a. NK	1	1	3
		Ps. a. MO	1	1·5	2
		Ps. f.	0·5	1	1

Legend as in Table II.

At 1–3 per cent urea concentrations, isoleucine formation was observed up to 4–5 mg/ml in media containing 1 per cent α–ABA and 6–7 mg/ml in media containing 2 per cent α-ABA.

Of the bacteria employed, the strains of *Pseudomonas aeruginosa* were in general better than those of *Pseudomonas fluorescens*.

3. *Effect of ammonium salts.* In the experiments (1) and (2) described above, urea was employed as ammonia source. However, it is quite possible that the formation of isoleucine is influenced by the kind of ammonia sources, so that to choose the most favourable ammonium salt, media which contained

ammonium chloride, ammonium phosphate, ammonium nitrate, ammonium sulphate and urea, were compared. Concentrations of the respective ammonium salts were equalized to 1 per cent urea on a nitrogen basis.

Table IV. Effect of urea concentration on the accumulation of L-isoleucine

Medium	α-Amino-butyric acid (%)	Urea (%)	Strain	L-Isoleucine formed (mg/ml) Days of culture		
				2	3	4
AB–13	1	0	Ps. a. NK	0·5	1	1
			Ps. a. MO	0·5	0·5	0·5
			Ps. f.	0·5	1	1
AB–4	1	0·5	Ps. a. NK	1	2	1·5
			Ps. a. MO	1·5	2	2
			Ps. f.	1·5	1·5	2
AB–14	1	1	Ps. a. NK	3	4	2·5
			Ps. a. MO	3	4	4
			Ps. f.	2	3	2·5
AB–15	1	2	Ps. a. NK	2	3	2·5
			Ps. a. MO	3	4	3
			Ps. f.	2	3	2
AB–16	1	3	Ps. a. NK	2	3·5	3
			Ps. a. MO	3	5	4
			Ps. f.	1	2	1·5
AB–17	1	5	Ps. a. NK	1	3·5	2·5
			Ps. a. MO	1	3	1·5
			Ps. f.	0	0	1
AB–18	2	1	Ps. a. NK	1·5	3	5
			Ps. a. MO	2	3	6
			Ps. f.	2	3	4
AB–19	2	2	Ps. a. NK	1	2	4
			Ps. a. MO	4	7	6
			Ps. f.	2	3	4
AB–20	2	3	Ps. a. NK	1	3	4
			Ps. a. MO	2	3	3·5
			Ps. f.	1	2	2

Legend as in Table II.

Table V. Effect of ammonia source on the accumulation of L-isoleucine

Medium	Ammonia source	Strain	L-Isoleucine formed (mg/ml) Days of culture			
			2	3	4	5
AB–14	$(NH_2)_2CO$	Ps. a. NK	2	2	3	2·5
		Ps. a. MO	3	3	3	2
		Ps. f.	1	1·5	2·5	2
AB–21	NH_4Cl	Ps. a. NK	1	1·5	2	2
		Ps. a. MO	1·5	2	2	2
		Ps. f.	1	1·5	1·5	2
AB–22	$(NH_4)_2HPO_4$	Ps. a. NK	0	0	0	0
		Ps. a. MO	0	0·5	0·5	0·5
		Ps. f.	0	0	0	0
AB–23	NH_4NO_3	Ps. a. NK	0	0	0	0
		Ps. a. MO	0	0	0	0
		Ps. f.	0	0	0	0
AB–24	$(NH_4)_2SO_4$	Ps. a. NK	1	1	2	1·5
		Ps. a. MO	1·5	1	1	1
		Ps. f.	1	1	2	2

Legend as in Table II.

As indicated in Table V, urea gave the most favourable result among the salts employed and ammonium chloride and ammonium sulphate followed. In the media containing ammonium phosphate and ammonium nitrate, the growth of the organisms was retarded and almost no isoleucine formation occurred. Almost all the organisms employed showed the same effect of ammonia sources.

4. *Effect of organic nitrogen sources.* In order to investigate the most effective organic nitrogen sources for isoleucine formation, experiments were carried out on the media AB–25 to AB–33 (see Table I). The sources were added to the media to give a concentration of 0·1 per cent nitrogen. The results obtained are shown in Table VI.

Table VI. Effect of organic nitrogen sources on the accumulation of L-isoleucine

Medium	Organic nitrogen source	Strain	L-Isoleucine formed (mg/ml) Days of culture			
			2	3	4	5
AB–25	Casein hydrolyzate	Ps. a. NK	0	0	0	0
		Ps. a. MO	0·5	1	1	0·5
		Ps. f.	0	0	0	0
AB–26	Cornsteep liquor	Ps. a. NK	2	3	4	4
		Ps. a. MO	1·5	3	4	3·5
		Ps. f.	2	4	4	4
AB–27	Peptone	Ps. a. NK	1	2	4	4
		Ps. a. MO	2	3	3	4
		Ps. f.	1	2	2	2
AB–28	Yeast extract	Ps. a. NK	1	2	3	3·5
		Ps. a. MO	1·5	2·5	2	2·5
		Ps. f.	1	3	3·5	4
AB–29	Meat extract	Ps. a. NK	1	2·5	3	1·5
		Ps. a. MO	1	2	2	1
		Ps. f.	1	2·5	3·5	4
AB–30	Peptone + Yeast extract	Ps. a. NK	1	2	2	2
		Ps. a. MO	2	2·5	3	3
		Ps. f.	1	3	3	4
AB–31	Peptone + Meat extract	Ps. a. NK	1·5	3	4	4
		Ps. a. MO	1	1·5	2·5	2
		Ps. f.	1·5	3	4	3
AB–32	Soybean meal	Ps. a. NK	0	0	2	2
		Ps. a. MO	1	2	2	1
		Ps. f.	0	0	2·5	3
AB–33	Gelatin hydrolyzate	Ps. a. NK	1	2	2	3
		Ps. a. MO	1	2	2	1
		Ps. f.	1	2	2	1·5

Legend as in Table II.

In the medium containing cornsteep liquor, better growth of organisms was observed, and faster and higher formation of isoleucine occurred. Besides this, peptone, yeast extract, meat

extract and mixtures of these also gave favourable results. In casein hydrolyzate, although α-ABA was consumed as were the other compounds, very little isoleucine formation was observed.

No marked difference among the organisms employed of the effect of organic nitrogen sources was observed.

5. *Effects of α-aminobutyric acid concentration and shaking.* From the above results, cornsteep liquor and peptone were chosen as organic nitrogen source, and the effect of shaking was studied at varied concentrations of α-ABA (1–3 per cent).

The results, shown in Table VII, indicate that cornsteep liquor is generally more advantageous than peptone, and stationary culture was inferior to shaking culture.

Table VII. Effects of shaking and α-aminobutyric acid concentration on the accumulation of L-isoleucine

Medium	α-Amino-butyric acid (%)	Organic nitrogen source	Method of culture	Strain	L-Isoleucine formed (mg/ml) Days of culture			
					3	4	5	6
AB–34	1	Peptone	Shaking	Ps. a. NK	0	1	4	4
				Ps. a. MO	2	3·5	5	5
				Ps. f.	2	3	4	4
			Stationary	Ps. a. NK	0	1	1	1
				Ps. a. MO	1·5	2·5	2·5	4
				Ps. f.	1	2·5	2·5	2·5
AB–35	1	Cornsteep liquor	Shaking	Ps. a. NK	3	4	5	5
				Ps. a. MO	3	5	7	6
				Ps. f.	3	5	5	4
			Stationary	Ps. a. NK	0	0	1	2
				Ps. a. MO	1	2	2	2·5
				Ps. f.	0	1·5	2	2·5
AB–36	2	Peptone	Shaking	Ps. a. NK	0	1·5	2·5	3
				Ps. a. MO	4	6	5	5
				Ps. f.	0	0	1	1
			Stationary	Ps. a. NK	0	0	0·5	1
				Ps. a. MO	2·5	2	1·5	1·5
				Ps. f.	0	0	0·5	0

Table VII. *Continued*

AB–37	2	Cornsteep liquor	Shaking	Ps. a. NK	1	3	5	6
				Ps. a. MO	4	6	9	9
				Ps. f.	5	6	5	5
			Stationary	Ps. a. NK	0	0	0	0
				Ps. a. MO	1·5	2	3·5	4
				Ps. f.	2·5	3	4	4
AB–38	3	Peptone	Shaking	Ps. a. NK	0	0	0	0
				Ps. a. MO	2	2	2	3
				Ps. f.	0	0	0	0
			Stationary	Ps. a. NK	0	0	0	0
				Ps. a. MO	0	0·5	1	0·5
				Ps. f.	0	0	0	0
AB–39	3	Cornsteep liquor	Shaking	Ps. a. NK	0	0	1	1
				Ps. a. MO	2	5	8	9
				Ps. f.	1	1·5	3	3
			Stationary	Ps. a. NK	0	0	0	0
				Ps. a. MO	0·5	1	2	3·5
				Ps. f.	0·5	1	1	1

Legend as in Table II.

Higher concentration of α-ABA retarded the growth of organisms, and isoleucine formation at 3 per cent α-ABA was less than that at 2 per cent α-ABA. *Pseudomonas aeruginosa* NK especially, cultured on media containing 3 per cent α-ABA, showed very poor growth and no accumulation of isoleucine.

From these experiments, *Pseudomonas aeruginosa* MO was advantageous for isoleucine formation.

6. *Effect of cornsteep liquor concentration.* The experiments (4) and (5) revealed the advantage of employing cornsteep liquor as organic nitrogen source, so that the effect of cornsteep liquor concentration was investigated with media AB–40 to AB–53.

As indicated in Table VIII, in media containing 0·55 per cent cornsteep liquor, growth of *Pseudomonas aeruginosa* NK was not observed in the presence of 2 per cent or 3 per cent α-ABA, and poor growth and inferior isoleucine formation also appeared in other strains employed.

Table VIII. Effect of cornsteep liquor concentration on the accumulation of L-isoleucine

Medium	Glucose (%)	α-Amino-butyric acid (%)	Corn-steep liquor (%)	Strain	L-Isoleucine formed (mg/ml) Days of culture			
					3	4	5	6
AB–40	10	2	0·55	Ps. a. NK	0	0	0	0
				Ps. a. MO	4	7	7	5
				Ps. f.	0	0	3	2
AB–41	10	2	1·1	Ps. a. NK	0	0	3	3
				Ps. a. MO	7	7	6	5
				Ps. f.	5	5	5	5
AB–42	10	2	2·2	Ps. a. NK	2	4	5	6
				Ps. a. MO	8	9	9	9
				Ps. f.	5	6	6	6
AB–43	10	2	4·4	Ps. a. NK	5	8	10	8
				Ps. a. MO	5	7	9	8
				Ps. f.	6	8	7	6
AB–44	10	3	0·55	Ps. a. NK	0	0	0	0·5
				Ps. a. MO	0	0	0	0
				Ps. f.	0	0	0	0
AB–45	10	3	1·1	Ps. a. NK	0	0	0	0·5
				Ps. a. MO	2·5	4	9	9
				Ps. f.	0	0	0	0·5
AV–46	10	3	2·2	Ps. a. NK	1	1·5	1·5	1
				Ps. a. MO	5	9	10	10
				Ps. f.	2·5	3	3	3·5
AB–47	10	3	4·4	Ps. a. NK	0	1·5	6	8
				Ps. a. MO	5	6	8	8
				Ps. f.	5	5	5	6
AB–48	15	2	0·55	Ps. a. NK	0	0	0	0·5
				Ps. a. MO	4	8	8	8
				Ps. f.	0	0	0	0·5
AB–49	15	2	1·1	Ps. a. NK	1·5	2	3	3
				Ps. a. MO	9	10	12	14
				Ps. f.	3	5	6	6
AB–37	15	2	2·2	Ps. a. NK	0	3	7	8
				Ps. a. MO	6	9	13	11
				Ps. f.	4	7	6	6

Table VIII. *Continued*

AB–50	15	2	4·4	Ps. a. NK	2	3	6	8
				Ps. a. MO	4	7	8	7
				Ps. f.	4	7	7	7
AB–51	15	3	0·55	Ps. a. NK	0	0	0	0·5
				Ps. a. MO	0	0	0	0
				Ps. f.	0	0	0	0
AB–52	15	3	1·1	Ps. a. NK	0	0·5	0·5	0·5
				Ps. a. MO	2.5	7	8	8
				Ps. f.	0	0·5	0·5	0
AB–39	15	3	2·2	Ps. a. MK	1	1	1	1·5
				Ps. a. MO	7	10	12	10
				Ps. f.	4	4	4	3·5
AB–53	15	3	4·4	Ps. a. NK	0	0	0·5	3
				Ps. a. MO	7	7	8	8
				Ps. f.	6	6	8	7

Legend as in Table II.

In media containing more than 1·1 per cent cornsteep liquor, these retardations of growth and isoleucine formation due to the higher concentration of α-ABA were somewhat mitigated. However, more isoleucine was accumulated in media containing 2 per cent α-ABA than in those with 3 per cent α-ABA.

Under the conditions tested, better results were generally obtained with *Pseudomonas aeruginosa* MO, which produced up to 13–14 mg/ml of L-isoleucine in media containing 15 per cent glucose and 1·1–2·2 per cent cornsteep liquor.

7. *Effect of strength of shaking.* In experiment (5) carried out in test tubes, shaking culture gave better results than stationary culture. The effect of shaking was further studied in shaking flasks by employing medium AB–37 which from the previous experiments seemed most favorable for isoleucine accumulation.

Besides the change of reciprocation rate, strength of shaking was also varied by the amounts of medium in the shaking flasks. From the results, shown in Table IX, it was confirmed that rather small amounts of medium, in other words, stronger shaking, favours isoleucine accumulation.

Table IX. Effect of strength of shaking on the accumulation of L-isoleucine

Reciprocating shaking c/min.	Amount of medium* (ml)	Strain	L-Isoleucine formed (mg/ml) Days of culture			
			3	4	5	6
90	25	Ps. a. NK	0	0·5	3	6
		Ps. a. MO	4	8	11	12
		Ps. f.	4	9	10	10
90	50	Ps. a. NK	0	0	2	4
		Ps. a. MO	3	6	6	7
		Ps. f.	2·5	7	6	7
90	100	Ps. a. NK	0	0	1·5	3
		Ps. a. NO	2	3	3	3·5
		Ps. f.	2	2	3	3·5
90	200	Ps. a. NK	0	0	0·5	1
		Ps. a. MO	0·5	1	1·5	1·5
		Ps. f.	0·5	0·5	0·5	1
110	25	Ps. a. NK	4	8	10	10
		Ps. a. MO	6	11	11	6
		Ps. f.	8	6	5	3
110	50	Ps. a. NK	4	6	10	10
		Ps. a. MO	5	11	10	11
		Ps. f.	7	6	5	4
110	100	Ps. a. NK	1	3	3	3
		Ps. a. MO	3·5	4·5	4	4
		Ps. f.	4·5	5	5	4

Fermentation was carried out with medium AB–37 at 37°.
* Amount (ml) of medium in 500–ml shaking flasks.

8. *Effects of inoculum size and temperature.* Effect of inoculum size was studied with *Pseudomonas aeruginosa* MO in medium AB–37. Isoleucine formation in the early stages of fermentation was somewhat promoted by three- or ten-fold increase of the inoculum size, although the maximum amount of isoleucine accumulated was not changed.

Effect of temperature was also studied, and it was ascertained that by incubation at 37° early isoleucine formation was slightly promoted but increase of accumulated isoleucine was not observed. Consequently, fermentation at 30° is preferable.

9. *Effect of time of additions of α-aminobutyric acid and glucose.* As revealed in the above mentioned experiments, concentrations of α-ABA and glucose in the media influence isoleucine accumulation. It was also possible that the formation is influenced by the time of addition of these substances.

Thus the time of additions of α-ABA and glucose to medium AB–37 was varied as 0, 8 and 48 h from the beginning of the fermentation as indicated in Table X.

Table X. Effect of time of addition of α-aminobutyric acid and glucose on the accumulation of L-isoleucine

α-Aminobutyric acid added (%)			Glucose added (%)		L-Isoleucine formed (mg/ml)				
Time of additions (h)					Days of culture				
0	8	48	0	48	2	3	4	5	6
2	—	—	15	—	3·5	9	11	9	7
2	—	—	10	5	4·5	10	12	5	0
—	2	—	15	—	3	9	10	6	3
—	2	—	10	5	4	11	10	4	1
—	—	2	15	—	0	1·5	2·5	2	1·5
—	—	2	10	5	0	0·5	0·5	0·5	0·5
1	1	—	15	—	3	9	10	8	7
1	1	—	10	5	4·5	10	6	2	0
1	—	1	15	—	3·5	7	6	3	0
1	—	1	10	5	2·5	2	1	1	0

Shaking culture (110 c/min at 30°) was carried out with *Pseudomonas aeruginosa* MO in the medium AB–37.

Either addition of all the α-ABA at the beginning of the fermentation or separate additions at the beginning and at 8 h gave the best accumulation of isoleucine: addition of α-ABA after 48 h gave almost no L-isoleucine accumulation. Accordingly, the presence of α-ABA at the growth phase of the organisms is considered to be required for the accumulation of L-isoleucine.

Separate addition of glucose, that is, 10 per cent at the beginning and 5 per cent 48 h later showed a tendency to form isoleucine faster than the addition of 15 per cent glucose from the beginning.

10. *Changes during fermentation.* The data for a typical L-isoleucine fermentation are given in Fig. 1. Shaking culture (110 c/min) was carried out with *Pseudomonas aeruginosa* MO in a 500-ml shaking flask containing 50 ml of medium AB–37, and formation of L-isoleucine, consumption of α-ABA and glucose, and change of pH were determined.

Maximum accumulation of L-isoleucine and the highest conversion rate from α-ABA were attained by 4 days incubation.

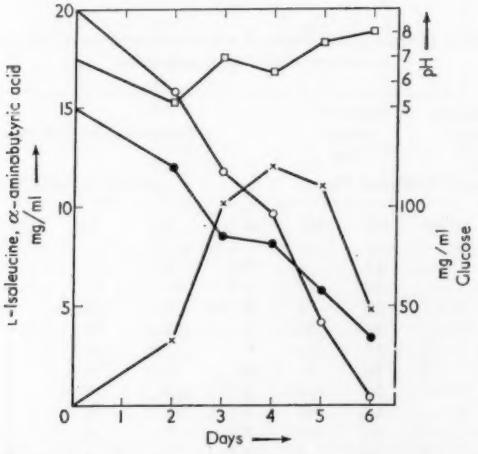


Fig. 1. Changes during Isoleucine Fermentation by *Pseudomonas aeruginose* MO.

Although consumption of α-ABA and glucose continued even after 4 days, increase of isoleucine was not promoted by prolonged incubation, which rather decreased the accumulated isoleucine.

Amino acids, other than L-isoleucine, produced in this fermentation are a very small amount of alanine and glutamic acid. Consequently, it is possible to isolate L-isoleucine from the filtered broth by the ordinary separation procedure employing ion-exchange resins.

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